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Changes in Menaquinone Composition Associated with Growth Phase and Medium Composition in *Amycolatopsis* Species

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Remarkable quantitative changes in menaquinone composition associated with the growth phase were found in the cells of *A. orientalis*. The bacterium produced an unsaturated menaquinone with nine isoprene units (MK-9) and its dihydrogenated MK-9 (H_2) menaquinone as major homologues at the early growth phase in the liquid media both of yeast extract-dextrose and brain heart infusion broth. However, at the middle to late exponential phases, the major component was changed to tetrahydrogenated menaquinone [MK-9(H_4)] . Such changes were medium-dependent and not observed with yeast extract-malt extract-dextrose medium. MK-9(H_4) was a major component throughout the growth phase in the medium.

Key words : menaquinone composition, variation, growth phase, medium composition

INTRODUCTION

Menaquinone is an essential coenzyme of the respiratory electron transport system in bacteria and its compositional variation has been used as one of the important chemotaxonomic criteria in the classification of bacteria (1, 2, 3, 11). The reason for its usefulness as a taxonomic character is due to the perceived homogeneity in the same taxon (2, 3).

During our studies on the menaquinone composition of some strains of *Amycolatopsis* species (8), it was found that these compositions varied depending on the growth phase and the medium used. In this paper, it is reported that the menaquinone composition of *A. orientalis*, especially the degree of hydrogenation, showed dependent changes. The results using 6 other *Amycolatopsis* spp. are also discussed.

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MATERIALS AND METHODS

Microorganisms. *A. orientalis* subsp. *orientalis* IFM 0528 (=ISP 5040=JCM 4600), *A. orientalis* subsp. *lurida* JCM 3141^T(T, type strain), *A. sulphurea* JCM 3142^T, *A. mediterranei* JCM 4789^T, *A. methanolica* JCM 8087^T, *A. fastidiosa* JCM 3276^T, *A. azurea* JCM 3275^T, and *A. rugosa* IFO 14506^T were used. The spores and mycelial fragment suspension were stored in 35% glycerol solution until use.

Growth conditions. The strains were grown in shake flasks of brain heart infusion broth (BHI, Difco) with 2.5% glucose for 3 to 4 days at 32°C and used for seed culture. Three liquid media were used for culturing the organism : Y-G medium (1.5% yeast extract and 1.5% glucose, pH 7.2), G-BHI (BHI medium with 2% glucose) and YMG medium (0.4% yeast extract, 1.0% malt extract, 0.4% glucose, pH 7.2). Y-G medium with different con-

centration of glucose was used as the basal medium to test the effect of glucose concentration on menaquinone composition. The seed cultures (final concentration, 2.5%) were inoculated into a 500 m*l* Erlenmeyer shake flask containing 200 m*l* of each medium. They were incubated at 32°C for 3, 6, and 8 days (for some strains, the incubation was continued for 14 days) at 250 rpm. Therefore, aliquots removed at intervals were centrifuged at 5,000 rpm for 10 min and the cultures were washed with distilled water three times. They were freeze-dried for extraction of menaquinones.

Growth phase determination. The growth phase was monitored by measuring the increase in dried biomass. The samples were removed and filtered through a cellulose nitrate membrane (0.22 μm pore size, Advantec, Japan). The filters were washed with distilled water, dried in an oven, and weighed.

Extraction of menaquinones. Dried cultures (about 100 mg) were extracted with 45 m*l* of a mixture of $CHCl_3$-methanol (2 : 1 v/v) overnight. The filtered extracts were dried in vacuo and a small amount of acetone was added to the extract. Menaquinone fractions were purified by preparative thin layer chromatography (Pre-TLC). The fraction was monitored by HPLC using the following conditions : column, Lichro cart RP-18 (Merck, 4.6×150 mm), flow rate, 1 m*l*/min ; detection, UV 235 nm; and mobile phase, methanol-iso-propanol (2 : 1). Components were identified by comparison of retention times with those of standard mixtures whose structures were determined by mass spectrometry.

Structural determination. Mass spectrometry was performed by a Hitachi M-60 spectrometer using a direct insertion probe, a chamber at 240°C and a sample at 230°C. The menaquinone structures were deduced from the fragmentation patterns. To confirm the structures, ^1H spectrum was measured on a JEOL-500 Alpha NMR spectrometer at 500 MHz and compared with those of authentic menaquinone samples.

Reproducibility. The reproducibility of the menaquinone profiles is believed to be influenced by extraction and analysis conditions, so the experiments were conducted two or three times.

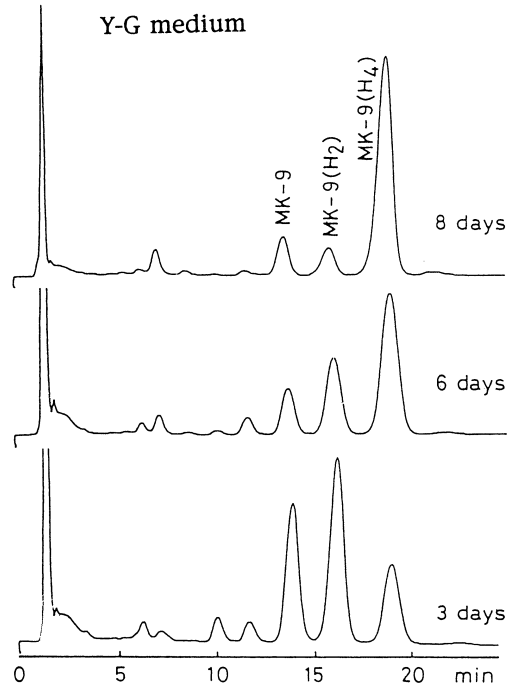

Fig. 1. HPLC elution profiles of menaquinone fractions from *A. orientalis* subsp. *orientalis* IFM 0528 grown in Y-G medium
Numbers indicate period of incubation. See Materials and Methods for analytical conditions

RESULTS

Since our preliminary studies indicated that the menaquinone compositions vary depending on the sample of *A. orientalis*, the menaquinones were first extracted from the cultures of different growth phases of *A. orientalis* subsp. *orientalis* IFM 0528. As shown in Fig. 1, it was found that the menaquinone composition of this subspecies changed in response to the growth phase in Y-G medium. On day 3, MK-9 and MK-9(H_2) were the major menaquinones, and MK-9(H_4) was present as a minor menaquinone. However, with progress of the incubation period, the proportion of MK-9(H_4) increased, and by day 6 about 40 to 50% of the menaquinones had turned into MK-9(H_4). By day 8 about 80% had become MK-9(H_4) (Table 1). A

Table 1. Effect of medium ingredient and incubation time on menaquinone compositions in 7 species (including one subspecies) of *Amycolatopsis*

Species	Medium	Day	Menaquinone isoprenologue (%)		
			9(H_0)	9(H_2)	9(H_4)
A. orientalis subsp. *orientalis* IFM 0528	Y-G	3	31	46	23
		6	2	27	71
		8	11	10	79
	G-BHI	3	38	43	19
		6	23	44	33
		8	18	24	58
	YMG	3	2	8	90
		6	2	3	95
		8	2	2	96
A. orientalis subsp. *lurida* JCM 3141^T	Y-G	3	7	41	52
		6	9	21	70
		8	7	13	80
	G-BHI	3	18	40	42
		6	13	41	46
		8	10	31	59
A. sulphurea JCM 3142^T	Y-G	3	ND	ND	100
		6	ND	ND	100
		8	ND	ND	100
	G-BHI	3	ND	ND	100
		6	ND	ND	100
		8	ND	ND	100
A. mediterranei JCM 4789^T	Y-G	3	6	12	82
		6	8	7	85
		8	8	8	84
	G-BHI	3	8	7	85
		6	9	8	84
		8	8	8	84
A. methanolica JCM 8087^T	Y-G	3	6	36	58
		6	7	8	85
		8	2	3	95
	G-BHI	3	9	23	68
		6	7	8	85
		8	8	7	85
A. rugosa IFO 14506^T	Y-G	3	ND	ND	100
		6	ND	ND	100
		8	ND	ND	100
	G-BHI	3	ND	ND	100
		6	ND	ND	100
		8	ND	ND	100
A. fastidiosa JCM 3276^T	Y-G	3	ND	ND	100
		6	ND	ND	100
		8	ND	ND	100
	G-BHI	8	5	11	84
		10	4	6	90
		14	27	ND	73
A. azurea[a)] JCM 3275^T	YMG	3	9	10	81
		6	7	3	90
		8	7	3	90

[a)] Since the growth in Y-G and G-BHI was extremely slow, YMG medium was used

ND : not detected

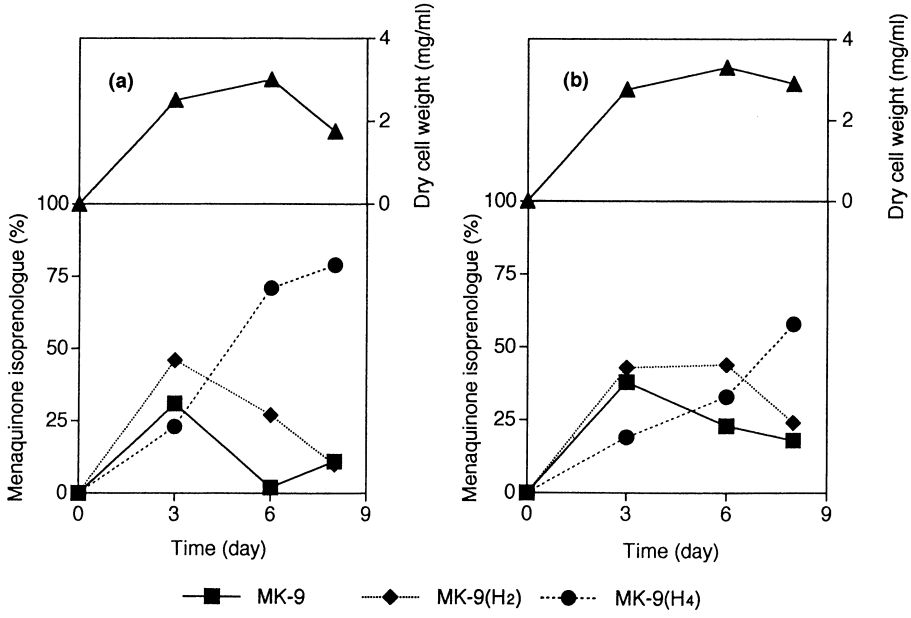

Fig. 2. Changes in menaquinone composition of *A. orientalis* subsp. *orientalis* IFM 0528 associated with time course of growth in (a) Y-G medium and (b) G-BHI medium

very similar time course of these compositions was observed in G-BHI medium.

In contrast, however, as shown in Table 1, such growth phase dependent variations of the compositions were not observed in YMG medium. Throughout the growth phase, the major component observed was MK-9(H_4).

Whether such medium-dependent changes in menaquinone composition is a common phenomenon in *Amycolatopsis* species was also tested . The menaquinones were extracted, and their compositions were determined using six species of *Amycolatopsis* (Table 1). Among seven tested species of *Amycolatopsis, A. orientalis* subsp. *lurida* JCM 3141^T showed a change of menaquinone composition similar to that of *A. orientalis* subsp. *orientalis* IFM 0528. Such a remarkable variation in composition was not observed with other *Amycolatopsis* species, however, although moderate quantitative changes in composition were also confirmed in *A. methanolica* JCM 8087^T and *A. fastidiosa* JCM 3276^T (on day 14).

The association of the growth phase of *A. orientalis* subsp. *orientalis* IFM 0528 with menaquinone composition was studied using Y-G and G-BHI. On the basis of dry weight, it was found that the growth on day 3 was late log phase or early stationary phase (Fig. 2). On day 6, the cultures reached maximum growth in both media, and then the biomass began to decrease. The initial pH (7.0) gradually decreased with the progress of the incubation period, and the lowest pH value was observed on day 3 ; thereafter, the pH began to increase and this increase continued even after day 8 (data not shown). The rate of MK-9(H_4) composition gradually increased with the progress of the incubation period. In contrast, the ratio of MK-9 and MK-9(H_2) increased up to day 3 and then declined.

Since glucose was contained commonly in the three media tested, we were interested in the effect of glucose concentration on the menaquinone composition in *A. orientalis* subsp. *orientalis* IFM 0528. As shown in Fig. 3, the ratio of MK-9 to the other two menaquinones was high when 1.5% glucose was used. As the glucose concentration was increased, the percent of MK-9(H_4) increased.

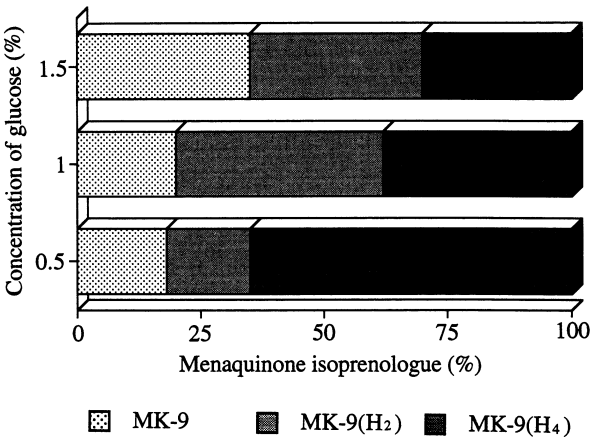

Fig. 3. Effect of glucose concentration on the menaquinone compositions of *A. orientalis* subsp. *orientalis* IFM 0528

The basal medium to which glucose was added Y-G medium. The cultures were harvested after 3 days to determine the menaquinone compositions

DISCUSSION

The length and degree of hydrogenation of the multiprenyl side chain have been used for classification and identification as important taxonomic characteristics because they are stable within strains of the same taxon (2, 3). Although actinomycetes contain complex mixtures of partially saturated menaquinones, such menaquinones have also been believed to be stable and considered to have important taxonomic characteristics.

Recently, however, observations have been made on the changes of menaquinone composition in the side chain length or degree of hydrogenation in some bacteria. Hammond et al. (7) reported that in *Staphylococcus aureus*, the proportions of the menaquinone homologues changed during the exponential growth phase. Hiraishi et al. (5, 6) also reported that *Micrococcus varians* IAM 12146 produces different quinones, namely, menaquinones and demethylmenaquinones depending on the medium composition used. Furthermore, they also indicated that the demethylmenaquinone/menaquinone ratio in cells varied during growth. Saddler et al. (10) reported that the isoprenoid quinone composition depended on the growth phase in *Streptomyces cyaneus*, although the fatty acid profiles remained relatively constant during its growth phase. In the present experiment using *Amycolatopsis*, one of the genera of actinomycetes, changes in menaquinone composition depend on the growth phase and medium composition were also confirmed. Throughout the present studies, it was clear that this variation is significantly influenced by the stage of culture. In the early stage, the predominant menaquinones were MK-9(H_0) and MK-9(H_2), and with the progress of the incubation period, the major menaquinone became MK-9(H_2) and, finally, MK-9(H_4). These data seem to indicate that the degree of hydrogenation in the isoprenoid side chain proceeds toward saturation, coupled during the incubation period. Therefore, it is recommended that relatively old cultures of the stationary phase be used for the determination of menaquinone composition. Particularly, remarkable variation of these compositions was observed in the special species *A. orientalis* subsp. *lurida* JCM 3141^T and *A. orientalis* subsp. *orientalis* IFM 0528 among those tested. Henssen et al. (4) reported that most *Amycolatopsis* species have MK-9(H_2) menaquinone in addition to MK-9(H_4). According to Merz and Yao (9), although the major menaquinone of *Amycolatopsis alba* is MK-9(H_4), MK-8(H_4) is present as a minor menaquinone. Therefore, these variations in menaquinone composition may be a species or strain-specific phenomenon.

In this experiment, the effect of glucose on the menaquinone composition was studied, and it was found that glucose content appears to have an impact on this composition. Although the reason is not clear at present, such variation may partially be due to the growth phase of the culture because glucose is a limiting factor for growth.

The present studies made it clear that the description of the culture condition, including medium composition, is necessary to avoid confusion and to obtain reproducible results in menaquinone composition analysis.

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Amycolatopsis 属細菌の生育期および培地組成によるメナキノン組成の多様性

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Amycolatopsis 属7種菌株のうち *A. orientalis* は生育段階に関連したメナキノン組成において顕著な組成の多様性が認められた．酵母エキス，グルコース培地やブレインハートインフュージョン培地のような液体培地で培養した場合，生育初期には MK-9 とその主要類似体として MK-9(H_2)を生産した．しかしながら生育の中期から後期では MK-9(H_4)へと変化した．このようなメナキノン組成の多様性は培地組成に依存しており，酵母エキス，麦芽エキス，グルコース培地では観察されなかった．本培地使用時では MK-9(H_4)が生育初期より主要メナキノンであった．