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An *Escherichia coli* mutant containing only demethylmenaquinone, but no menaquinone: effects on fumarate, dimethylsulfoxide, trimethylamine N-oxide and nitrate respiration

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Abstract. The mutant strain AN70 *(ubiE)* of *Escherichia coli* which is known to lack ubiquinone (Young IG et al. 1971), was analyzed for menaquinone (MK) and demethylmenaquinone (DMK) contents. In contrast to the wild-type, strain AN70 contained only DMK, but no MK. The mutant strain was able to grow with fumarate, trimethylamine N-oxide (TMAO) and dimethylsulfoxide (DMSO), but not with nitrate as electron acceptor. The membranes catalyzed anaerobic respiration with fumarate and TMAO at 69 and 74% of wild-type rates. DMSO respiration was reduced to 38% of wild-type activities and nitrate respiration was missing ($\leq$ 8% of wild-type), although the respective enzymes were present in wild-type rates. The results complement earlier findings which demonstrated a role for DMK only in TMAO respiration (Wissenbach et al. 1990). It is concluded, that DMK (in addition to MK) can serve as a redox mediator in fumarate, TMAO and to some extent in DMSO respiration, but not in nitrate respiration. In strain AN70 *(ubiE)* the lack of ubiquinone (Q) is due to a defect in a specific methylation step of Q biosynthesis. Synthesis of MK from DMK appears to depend on the same gene *(ubiE)*.

Key words: Demethylmenaquinone – Menaquinone – Anaerobic respiration – Fumarate respiration – Nitrate respiration – *Escherichia coli*

Escherichia coli contains 3 different quinones, ubiquinone (Q), menaquinone (MK) and demethylmenaquinone (DMK), which are produced in variable amounts depending on the growth conditions (Bentley and Meganathan 1987; Unden 1988). The quinones serve as redox mediators in the respiratory electron transfer chains. The different functions of the quinones in enteric bacteria have first been studied by extracting and incorporating the quinones into bacterial membranes (Kröger et al. 1971). Investigations on the specific functions of the quinones have been greatly facilitated by the availability of mutants of *E. coli*, which were either deficient of Q (*ubiA* mutant) or of the naphthoquinones MK and DMK (*menA* mutant) (Guest 1977, 1979; Wallace and Young 1977b; Wissenbach et al. 1990; Kwan and Barrett 1983; Meganathan 1984). With these mutants it was demonstrated that Q serves as a redox mediator in aerobic and nitrate respiration while the naphthoquinones fulfill the same function in anaerobic respiration with nitrate, fumarate, DMSO and TMAO as the acceptors. *E. coli* always contains a mixture of the naphthoquinones MK and DMK and no mutant strain was available which produced either DMK or MK alone (Unden 1988). Therefore the relative contribution of MK and DMK to the function of the naphthoquinones in anaerobic respiration could not be easily tested so far. Incorporation of either the MK analogue vitamin K_1 or of DMK into membranes of *E. coli* AN386 *(menA)* indicated that MK activated fumarate, nitrate, TMAO and DMSO respiration. On the incorporation of DMK high rates only of TMAO respiration were observed (Wissenbach et al. 1990). Thus no specific role or requirement for DMK in anaerobic respiration could be recognized from the experiments. On the other hand, DMK is synthesized in relatively large amounts in the enteric bacteria (Unden 1988). This suggests a more important function of DMK. Therefore a mutant containing DMK in the absence of MK would be desirable to differentiate the specific roles of MK and DMK. Here it will be shown that an *E. coli* mutant (AN70 *ubiE*) which is deficient of a specific methylase of the Q biosynthetic pathway (Young et al. 1971), is completely devoid of MK and contains high levels of DMK. This strain was used to study the function of DMK in anaerobic respiration without interference from MK.

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Abbreviations: DMSO = dimethylsulfoxide; DMS = dimethylsulfide; TMAO = trimethylamine N-oxide; TMA = trimethylamine; BV = benzylviologen; BV_{red} = reduced benzylviologen; Q = ubiquinone; MK = menaquinone; DMK = demethylmenaquinone; NQ = naphthoquinone

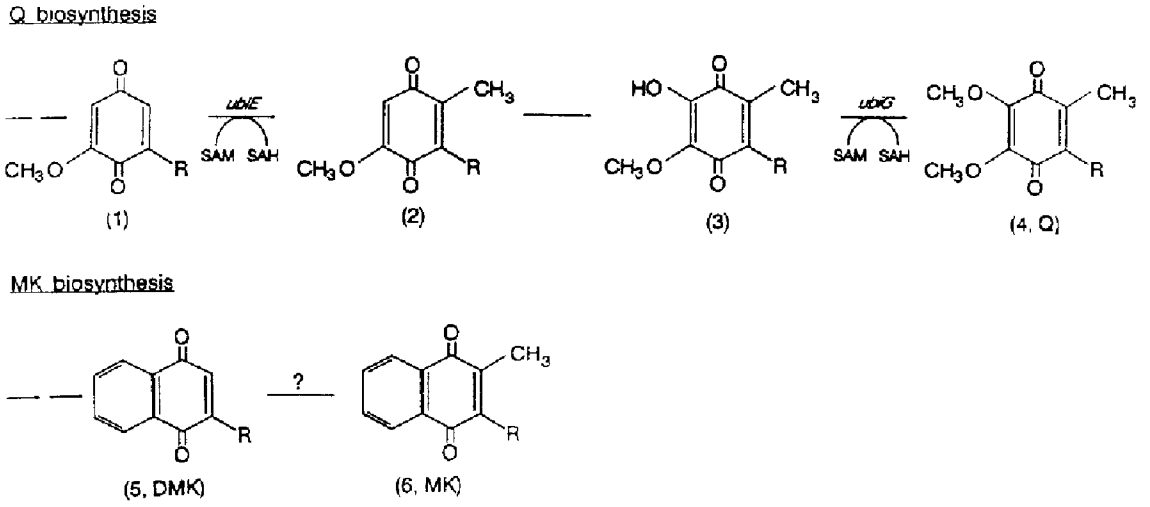

Fig. 1. Methylation reactions and intermediates in the pathways of Q and MK biosynthesis of *E. coli* (according to Bentley and Meganathan 1987). R = Octaprenyl; SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine; *ubiE, ubiG* = genetic loci in mutant strains. *1* = 2-octaprenyl-6-methoxy-1,4-benzoquinone; *2* = 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone; *3* = 2-octaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinone; *4* = 2-octaprenyl-3-methyl-5,6-methoxy-1,4-benzoquinone (Q); *5* = 2-octaprenyl-1,4-naphthoquinone (DMK); *6* = 2-octaprenyl-3-methyl-1,4-naphthoquinone (MK)

Methods

Bacteria and growth

Escherichia coli strains AN387 (wild-type), AN386 (*menA* 401), AN385 (*ubiA* 420), AN151 (*ubiG* 423, *metB*) and AN70 (*ubiE* 401, *metB*) were used for the experiments (Stroobant et al. 1972; Young et al. 1971; Wallace and Young 1977b). The strains were kindly supplied by I. G. Young, Canberra. The enzymatic reactions and intermediates of Q-biosynthesis which are affected in the different mutant strains are given in Fig. 1. For growth L-broth (Miller 1972) or the mineral salts medium M9 (Miller 1972) supplemented with a HCl-hydrolysate of casein (0.5 g/l type C/HSF, Serva, Heidelberg, FRG) and tryptophan (75 mg/l) were used (Wissenbach et al. 1990). The growth substrates were added in the following concentrations as indicated for each experiment: glucose 10 mM (aerobic growth) or 30 mM (anaerobic growth), glycerol (80 mM), Na_2-fumarate (50 mM), DMSO (50 mM), TMAO (50 mM), Na-nitrate (40 mM). For anaerobic growth the bacteria were grown in rubber stoppered glass bottles under an atmosphere of N_2 in degassed media. In anaerobic cultures used for the isolation of membranes for measurement of anaerobic respiration, the N_2-atmosphere was replaced by H_2 (10^5 Pa). For measurement of nitrate respiration the bacteria were grown with lactose (10 mM) and molybdate (1 µM) without further external acceptor to increase hydrogenase activity (Wissenbach et al. 1990). Aerobic growth was performed in agitated (200 rpm) Erlenmeyer flasks (1 l) containing 100 ml medium. The bacteria were grown at 37 °C to the late logarithmic or early stationary growth phase.

Extraction and analysis of quinones

The quinones were extracted from about 0.5 g wet weight of cells with petrol ether and acetone as described (Unden 1988). For analysis the quinones were separated by high-performance liquid chromatography on a reversed phase column (Lichrospher RP-18, 5 µm, 250 × 4 mm; Merck, Darmstadt, FRG) according to Kroppenstedt (1985) and Unden (1988). The quinones were quantified by measuring the absorption of the eluting bands at 270 nm or 245 nm. Q-8, DMK-8 and MK-8 of *E. coli* eluted after 7.8 min, 10.6 min and 11.8 min (flow 1 ml/min). A standard of vitamin K_1 and Q-10 was used for quantification. The concentration of the standards was determined photometrically as described by Kröger and Dadák (1969).

Enzyme activities and electron transfer rates

Fumarate, nitrate, TMAO and DMSO reductases were measured photometrically by the substrate dependent oxidation of reduced benzyl viologen (BV_{red}) in anaerobic cuvettes (Wissenbach et al. 1990). Hydrogenase activity was determined photometrically as the reduction of BV by H_2 (Unden et al. 1982). Electron transport with H_2 as the donor and fumarate, nitrate, TMAO and DMSO as the acceptors was measured with the H_2-electrode (Hansatech, Bachofer, Reutlingen, FRG) as described by Wissenbach et al. (1990). One unit of activity corresponds to 2 µmol BV_{red} or 1 µmol H_2-oxidized or 2 µmol BV reduced per min.

The theoretical or maximal electron transport rates V_{ET} in the mutant strains were calculated from the activities of hydrogenase (V_{Hyd}) and the terminal reductases (V_{Red}) according to the equation:

$$V_{ET} = C \times \frac{V_{Hyd} \times V_{Red}}{V_{Hyd} + V_{Red}}$$

as described by Wissenbach et al. (1990). The activities of the enzymes were determined with BV. The equation gives the maximal electron transfer rates which can be obtained with the respective activities of hydrogenase and the terminal reductases, provided that a suitable redox mediator (MK or Q in the wild-type) is present. V_{ET} corresponds to 100% for wild-type *E. coli* with MK or Q present. For the constant C the following values were used: C = 0.98 for fumarate, C = 0.62 for DMSO, C = 0.32 for TMAO and C = 0.46 for nitrate respiration (Wissenbach et al. 1990).

Other methods

Protein was measured by the biuret method with KCN (Bode et al. 1968). Membranes for measurement of enzymatic and electron transport activities were prepared as described (Wissenbach et al. 1990).

Results

Naphthoquinones in E. coli AN70 (ubiE) and AN151 (ubiG)

For the experiments strains of *Escherichia coli* were used which are either wild-type with respect to quinone

Table 1. Quinones in *Escherichia coli* strains grown anaerobically in M9 or L-medium with the substrates given. The quinones were extracted from the bacteria and quantified by HPLC-analysis Glyc = glycerol, Fum = fumarate, Wt = wild-type

Strain	Substrate	MK	DMK	MK + DMK	Q
		(nmol/g dry weight)			
M9-medium:					
AN70 *(ubiE)*	Glucose	≦0.4	80.5	80.7	0.2
	Glyc + Fum	≦0.4	178.5	178.9	2.1
	Glyc + NO_3^-	≦0.5	172	172	5.5
AN151 *(ubiG)*	Glucose	0.4	121.5	121.7	0.6
	Glyc + Fum	9.5	181	190.5	0.6
AN387 (Wt)	Glucose	154	57	243	74
	Glyc + Fum	98.5	68.5	167	91
AN386 *(menA)*	Glucose	≦0.3	≦0.3	≦0.4	212
L-medium					
AN70 *(ubiE)*	Glucose	≦1	143	144	≦1
AN151 *(ubiG)*	Glucose	38	115	152	≦5

synthesis (strain AN387) or completely deficient of the naphthoquinones (strain AN386 *menA*) or deficient of two different methylation steps of Q biosynthesis (strains AN70 *ubiE* and AN151 *ubiG*). The strains were analyzed for their contents of MK, DMK and Q (Table 1). As expected strain AN70 *(ubiE)* contained < 1 to 3% of the Q contents found in the wild-type grown under comparable conditions. In addition, MK was completely missing, but DMK was present in amounts comparable to those of the wild-type. This was observed for anaerobic growth with fermentable substrates (glucose) as well as nonfermentable substrates like glycerol plus fumarate, nitrate (Table 1), DMSO or TMAO (not shown). In all cases the contents of MK were below 0.5% of the contents of DMK and similar to the contents of MK in strain AN386 *(menA)*, which is deficient of both naphthoquinones. Growth in rich media (L-broth) did not increase the amount of MK. Surprisingly strain AN151 *(ubiG)*, which is defective in a different methylation step of Q biosynthesis (see Figure 1) also contained decreased levels of MK after anaerobic growth on glucose (Table 1). However, after growth of strain AN151 on nonfermentable substrates like glycerol plus fumarate as the acceptor or in L-broth significantly higher amounts of MK were detected, although the high contents of the wild-type were not achieved. Therefore it appears that the gene product of *ubiE*, which is responsible for a specific methylation step in Q biosynthesis, is essential for the methylation of DMK which results in MK. The gene product of *ubiG* might be involved in some way in the same reaction. With strains AN387 (wild-type), AN70 *(ubiE)* and AN386 *(menA)* therefore strains are available which contain either both naphthoquinones (strain AN387), only DMK in the absence of MK (strain AN70) or no naphthoquinone (strain AN386). With this set of strains it was possible to study the specific functions of DMK and MK in anaerobic respiration.

Anaerobic growth of E. coli AN70 (ubiE) on nonfermentable substrates

The operation of DMK in anaerobic respiration with fumarate, nitrate, TMAO or DMSO was studied by anaerobic growth of *E. coli* AN70 on the respective acceptors and glycerol as the donor. Growth was compared to that of the wild-type and the *menA* mutant strain AN386. Strain AN70 *(ubiE)* grew well on glucose as well as on the nonfermentable substrates with the exception of glycerol plus nitrate (Table 2). The final growth and doubling times ($t_d \approx 2$–4 h) were typical for anaerobic growth in defined media and very similar to those of the wild-type, whereas the *menA* mutant as a negative control did not grow on the nonfermentable substrates. With nitrate as the acceptor strain AN386, in contrast to AN70, was able to grow due to the presence of Q. Taken together the results indicate that AN70 containing only DMK but no MK and Q, is able to grow by fumarate, TMAO or DMSO respiration similar to the wild-type, but not by nitrate respiration.

Fumarate and DMSO respiration in membranes of E. coli AN70 (ubiE) containing only DMK

The electron transfer activities with the acceptors fumarate, DMSO, TMAO and nitrate were measured in the membrane fractions of bacteria grown with the respective acceptors. H_2 was provided as the donor of electron transport which was determined by recording the H_2 concentration using a H_2-electrode (Tables 3 and 4). By measuring the activities of the individual enzymes (hydrogenase and the respective terminal reductase) a theoretical maximal electron transport activity (H_2 → terminal acceptor) was calculated assuming that the electron transfer between hydrogenase and the reductase is not limited by the redox reactions of the quinone present (Wissenbach et al. 1990). The wild-type membrane contained Q, MK and DMK at high concentrations and the measured electron transfer rate corresponded to approximately 100% of the theoretical rate. A measured electron transfer

Table 2. Growth and doubling time of *Escherichia coli* AN70 *(ubiE)* under anaerobic conditions. The bacteria were grown in M9 medium with different growth substrates. The final growth (ΔA_{578}) was measured after 24 h. Wt = wild-type

Substrate	AN70 *(ubiE)*		AN387 (Wt)		AN386 *(menA)*	
	t_d(h)	A_{578}	t_d(h)	A_{578}	t_d(h)	A_{578}
Glucose	1.8	0.55	1.7	0.80	5.0	0.45
Glycerol + fumarate	3.3	0.68	3.5	0.74	≧8	0.10
Glycerol + NO_3^-	≧8	0.34	2.0	0.71	2.0	0.72
Glycerol + TMAO	2.5	0.78	3.3	0.74	≧8	0.13
Glycerol + DMSO	6.5	0.51	6.5	0.43	≧8	0.11

Table 3. Fumarate and DMSO respiration in membrane fractions of *E. coli* AN70 *(ubiE)*. The bacteria were grown anaerobically with H_2 ($10^5 \times$ Pa) in M9 medium with glucose plus fumarate or glucose plus DMSO, respectively, as the substrates. The activities of the individual enzymes were determined photometrically, the respiratory activities ($H_2 \rightarrow$ Fum or $H_2 \rightarrow$ DMSO) were determined with the H_2-electrode. % theor. ET gives the % of theoretical (maximal) electron transport which was calculated from the individual enzymes (hydrogenase and terminal reductase) according to the equation given in the 'Methods' section

Strain	Fumarate respiration				DMSO respiration			
	$BV_{red} \rightarrow$ Fum	$H_2 \rightarrow$ BV	$H_2 \rightarrow$ Fum		$BV_{red} \rightarrow$ DMSO	$H_2 \rightarrow$ BV	$H_2 \rightarrow$ DMSO	
	U/g	U/g	U/g	% theor. ET	U/g	U/g	U/g	% theor. ET
AN70 *(ubiE)*	1250	980	370	69	360	1110	64	38
AN387 (Wild-type)	1830	1240	702	97	315	1290	187	119
AN386 *(menA)*	1970	1510	25	3	495	1390	5	2

rate falling short of the theoretical value indicates a limitation in redox mediation by the quinone present (Wissenbach et al. 1990).

The membranes of the fumarate grown bacteria showed high activities of fumarate reductase and hydrogenase. Fumarate respiration in the membranes of strain AN70 which contained only DMK, amounted to 69% of the theoretical values and was therefore comparable to the wild-type. In the negative control AN386 (deficient of MK and DMK) only negligible electron transport could be measured. For DMSO respiration (Table 3) the results were similar for the wild-type and strain AN386 (119% and 2% of theoretical activities) although the overall rates were lower due to the low DMSO reductase activities. In the membranes containing only DMK (*E. coli* AN70) DMSO respiration amounted to only 38% of the rates which could be theoretically expected from the enzyme activities. This indicates that DMK, compared to MK, might be limiting in DMSO respiration.

TMAO and nitrate respiration in membranes of E. coli AN70 containing only DMK

In TMAO respiration the quinones behave very similar to fumarate respiration (Table 4). In strain AN70 the electron transfer amounted to 74% of the theoretical value indicating that DMK is an efficient redox mediator. Nitrate respiration, however, showed a different response to the quinones. In strains AN387 (wild-type), AN386 *(menA)* and AN385 *(ubiA)* (not shown) high activities were measured due to the presence of MK or Q. Membranes of AN70 catalyzed only low rates of electron transfer ($\leqq$ 8% of wild-type), similar to the rates (3%) measured in the double mutant AN384 *(menA ubiA)* lacking Q and both naphthoquinones (Wissenbach et al. 1990). This demonstrates that DMK, in contrast to Q and MK, does not operate effectively in nitrate respiration.

The redox mediator in fumarate, DMSO and TMAO respiration in the membranes of strain AN70 has to be DMK. The only other quinone present in significant amounts (25 nmol/g dry weight) (Young et al. 1971) is 2-octaprenyl-6-methoxy-1,4-benzoquinone (see Fig. 1). According to the data of Schnorf (1966) for this precursor a midpoint potential $E_0' \approx +200$ to 210 mV can be estimated. A quinone of this midpoint potential is too positive to act as a donor for fumarate, DMSO and TMAO reductases and has also been shown to be inactive in aerobic respiration (Wallace and Young 1977a).

Effect of growth rate and growth conditions on the relative amounts of MK and DMK

Previous experiments had indicated that synthesis of Q influences the proportion of MK relative to the total naphthoquinone content (Whistance and Threlfall 1968; Unden 1988). In order to study the effects of Q synthesis and growth rate on DMK methylation, *E. coli* strains AN385 *(ubiA)* and AN387 (wild-type) were grown with different substrates and in different media (Table 5). In the wild-type the proportion of MK (relative to the total naphthoquinone content) decreased slightly with decreasing growth rate during growth in mineral medium. The *ubiA* mutant (AN385) which lacks Q, generally contained higher proportions of MK than the wild-type and after anaerobic growth with glycerol plus fumarate more than

Table 4. TMAO and nitrate respiration in membrane fractions of *E. coli* AN70 *(ubiE)*. The bacteria were grown anaerobically in M9 medium with glucose plus TMAO or lactose under an atmosphere of H_2 (1.5×10^5 Pa). The respiratory activities and the activities of the enzymes were determined as described in Table 3

Strain	TMAO respiration				Nitrate respiration			
	$BV_{red} \rightarrow$ TMAO	$H_2 \rightarrow$ BV	$H_2 \rightarrow$ TMAO		$BV_{red} \rightarrow NO_3^-$	$H_2 \rightarrow$ BV	$H_2 \rightarrow NO_3^-$	
	U/g	U/g	U/g	% theor. ET	U/g	U/g	U/g	% theor. ET
AN70 *(ubiE)*	1220	2300	188	74	1675	1535	$\leqq$30	$\leqq$8
AN387 (Wild-type)	2850	4520	603	108	2510	1705	585	125
AN386 *(menA)*	3090	4150	$\leqq$25	$\leqq$4	2050	2415	480	94

Table 5. Effect of growth conditions on the relative amounts of the naphthoquinones MK and DMK in *Escherichia coli* AN387 (wild-type) and AN385 *(ubiA)*. The bacteria were grown in M9 medium or L-broth with the substrates given, harvested in the late logarithmic growth phase and analyzed for the quinones

Medium/substrate	AN387				AN385 *(ubiA)*		
	t_d	MK + DMK	Q	MK	MK + DMK	Q	MK
	(h)	(nmol/g)		MK + DMK	(nmol/g)		MK + DMK
M9/Glycerol + fumarate	3.5	157	57	0.43	190	≦1	0.77
M9/Glucose + fumarate	2	220	78	0.32	256	≦1	0.66
M9/Glucose + O_2	1	75	286	0.29	96	≦2	0.39
L/Glycerol + fumarate	0.8	187	124	0.67	220	≦2	0.86

three quarters of the naphthoquinones consisted of MK. After growth in rich medium both strains contained significantly higher relative amounts of MK. The results therefore would indicate that absence (AN385) or reduced (anaerobic growth of AN387) synthesis of Q might cause an increase in DMK methylation. The composition of the growth medium (mineral or rich medium) apparently, too, influences the methylation of DMK, whereas growth rate appeared to be of minor influence.

Discussion

Role of DMK in anaerobic respiration

The results presented suggest that DMK operates with nearly the same electron transport activity as MK in the respiration with fumarate or TMAO as acceptor, while it is less efficient with DMSO and nearly ineffective with nitrate. In agreement with these findings, $DMKH_2$ is suited by its midpoint potential (E'_0 $DMKH_2$/DMK = +36 mV) as an electron donor for fumarate (E'_0 fumarate/succinate = +30 mV), DMSO (E'_0 DMSO/DMS = +160 mV) and TMAO (E'_0 TMAO/TMA = +130 mV) respiration. The results are also in line with the growth rates of the mutant with the different acceptors (Table 2). The results therefore indicate that DMK participates in anaerobic respiration, but it is not essential for any of the anaerobic respiratory systems. With respect to fumarate respiration, *E. coli* appears to resemble *Haemophilus parainfluenzae*. This bacterium performs fumarate respiration with DMK, the only quinone present in the bacterium (Holländer 1976; Kroppenstedt and Mannheim 1989).

In previous experiments the role of MK and DMK was studied by incorporating either DMK or the MK-analogue vitamin K_1 into the membran fraction derived from an *E. coli* mutant lacking both naphthoquinones (Wissenbach et al. 1990). In these experiments DMK activated respiration with TMAO, but not with nitrate, in agreement with data presented here. In contrast to the results of this communication, however, respiration with fumarate or DMSO was not activated upon the incorporation of DMK, although hydrogenase, fumarate reductase and DMSO reductase were present in sufficiently large activities. This discrepancy may be caused by the treatment of the membrane fraction which is necessary for quinone incorporation.

Involvement of ubiE in Q and MK biosynthesis

The *E. coli* mutant strain AN70 *(ubiE)* was completely devoid of MK, whereas DMK was present in high amounts. The methylating reactions in Q and DMK biosynthesis (Fig. 1) so far have been studied only in cellfree extracts (for a review see Bentley and Meganathan 1987). The methylation of DMK is chemically very similar to that of 2-octaprenyl-6-methoxy-1,4-benzoquinone in Q biosynthesis, which depends on *ubiE*, since in both reactions the methyl group is inserted to a 1,4-quinoid ring systems at position C-3. Both reactions occur in association with the membrane at the prenylated precursors and S-adenosylmethionine is used as the methyl donor. These similarities suggest that the methylation of DMK is catalyzed by the enzyme encoded by *ubiE*. The reason for the partial inhibition of DMK methylation in strain AN151 *(ubiG)* is not known. A possible explanation might be a feedback inhibition of the ring methylase (presumably encoded by *ubiE*) by an intermediate accumulating in AN151 *(ubiG)*. An additional reason might be an insufficient supply of S-adenoylsmethionine during growth in mineral medium with some substrates (O_2, nitrate).

Ring methylases of other bacteria possibly show the same lack of specifity for the respective Q or MK precursor. Most species containing DMK and Q have been demonstrated to contain also MK in varying proportions. This applies to many, though not all, members of the family *Pasteurellaceae* (*Actinobacillus*, *Pasteurella* and *Haemophilus* species) as demonstrated recently (Kroppenstedt and Mannheim 1989; Mannheim et al. 1978).

A further indication for a methylase common to Q and MK biosynthesis comes from the effect of Q on MK synthesis in *E. coli*. Lack of Q (strain AN385) caused an increased portion of MK compared to the wild-type. Increased Q synthesis under aerobic conditions in wild-type *E. coli* on the other hand results in decreased proportion of MK. This can be explained by a competition of the DMK and Q precursors for the same methylase. Therefore in *E. coli* DMK has to be regarded as an intermediate of MK synthesis, which accumulates under some conditions due to a limiting step in biosynthesis, but not as a deliberately synthesized product.

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