JOURNAL OF BACTERIOLOGY, June 1987, p. 2862–2865
0021-9193/87/0602862-04$02.00/0

Vol. 169, No. 6

NOTES

Tetrahydrothiophene 1-Oxide as an Electron Acceptor for *Escherichia coli*

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Received 3 November 1986/Accepted 3 March 1987

Escherichia coli **used tetrahydrothiophene 1-oxide (THTO) as an electron acceptor for anaerobic growth with glycerol as a carbon source; the THTO was reduced to tetrahydrothiophene. Cell extracts also reduced THTO to tetrahydrothiophene in the presence of a variety of electron donors. Chlorate-resistant (*chl*) mutants (*chlA*, *chlB*, *chlD*, and *chlE*) were unable to grow with THTO as the electron acceptor. However, growth and THTO reduction by the *chlD* mutant were restored by high concentrations of molybdate. Similarly, mutants of *E. coli* that are blocked in the menaquinone (vitamin K_2) biosynthetic pathway, i.e., *menB*, *menC*, and *menD* mutants, did not grow with THTO as an electron acceptor. Growth and THTO reduction were restored in these mutants by the presence of appropriate intermediates of the vitamin K biosynthetic pathway.**

Facultative anaerobes such as *Escherichia coli* can grow either aerobically or fermentatively with glucose as a carbon source. In addition, these organisms can grow anaerobically on oxidizable substrates such as glycerol or lactate in the presence of an external electron acceptor. Although many compounds such as nitrate, nitrite, fumarate, trimethylamine oxide (TMAO), and dimethyl sulfoxide (DMSO) function as electron acceptors (2, 10–12, 20), the reductions of nitrate and fumarate are the most widely studied. Nitrate reduction requires the molybdenum-containing nitrate reductase, a quinone, and *b*-type cytochromes (10, 11). The quinone is normally ubiquinone, but menaquinone can substitute for it (11). In contrast, menaquinone is obligatory for the reduction of fumarate and TMAO (9, 15). Further, it is known that chlorate-resistant (*chl*) mutants which are defective in the synthesis or assembly of molybdenum cofactor are unable to grow with nitrate, TMAO, or DMSO (2, 10, 20).

In 1978, tetrahydrothiophene 1-oxide (THTO; Chemical Abstracts Service Registry no. 1600-44-8) was reported to stimulate the growth of an unidentified bacterium (19). We now show that THTO serves as an electron acceptor for the growth of *E. coli*. Roles for menaquinone and a molydbenum-containing enzyme in the reduction to tetrahydrothiophene (THT) by the THTO reductase system are defined.

(A preliminary account of these studies has been presented [J. Schrementi and R. Meganathan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, K128, p. 214].)

The *E. coli* strains tested are listed in Table 1. The minimal medium of Lambden and Guest (12) was supplemented by the addition of 0.1% Casamino Acids and 30 mg of tryptophan per liter. For complex medium, 0.4% peptone and 0.4% yeast extract were also added. The minimal and complex media contained either 0.3% glucose or 0.5% glycerol as the carbon source. THTO (Aldrich Chemical Co., Inc.) was added at a concentration of 25 mM. *o*-Succinylbenzoic acid (OSB) and 1,4-dihydroxy-2-naphthoic acid (DHNA) were prepared and used as described elsewhere (14, 15). Growth was monitored (Klett meter; no. 66 filter) as previously described (15). Large quantities of cells were obtained by growing *E. coli* at 37°C in a 2-liter flask filled to the top with complex medium. The cells were harvested in late log phase by centrifugation at 3,000 × *g* for 10 min. The cell pellet was washed twice by centrifugation and suspension in 400 ml of 0.02 M potassium phosphate buffer, pH 7. The pellet was kept frozen at −20°C.

For preparation of extracts, cells (3 g) were suspended in 5 ml of 0.02 M potassium phosphate buffer containing 5 mM dithiothreitol and 70 μM phenylmethylsulfonyl fluoride and passed through a French pressure cell twice at 16,000 lb/in^2. The extract was incubated with about 100 μg of DNase for 5 min at 30°C to reduce viscosity and centrifuged at 3,000 × *g* for 15 min, and the supernatant was used without further purification. The protein content of the extracts was determined by the method of Lowry et al. (13).

The incubation mixture in a total volume of 1 ml contained approximately 4 mg of cell extract protein and the following additional components (in micromoles): potassium phosphate buffer (pH 7), 200; dithiothreitol, 0.5; and THTO, 22. For assay with benzyl viologen and dithionite, 1.5 mg of protein was used. The electron donors were used in the following amounts (in micromoles): NADH, 5; NADPH, 5; sodium formate, 20; and sodium lactate, 40. Benzyl viologen in the amount of 0.2 μmol was reduced with 4 μmol of dithionite containing 8 μmol of $NaHCO_3$.

TABLE 1. *E. coli* strains used

Strain	Relevant genotype	Reference(s)
PL2024	Wild type	9, 16
JRG862	*menC*	9, 16
JRG918	*menD*	9, 16
JRG962	*menB*	16
382	*chlA*	1
442	*chlB*	1
C122	*chlD*	1
C26	*chlE*	1

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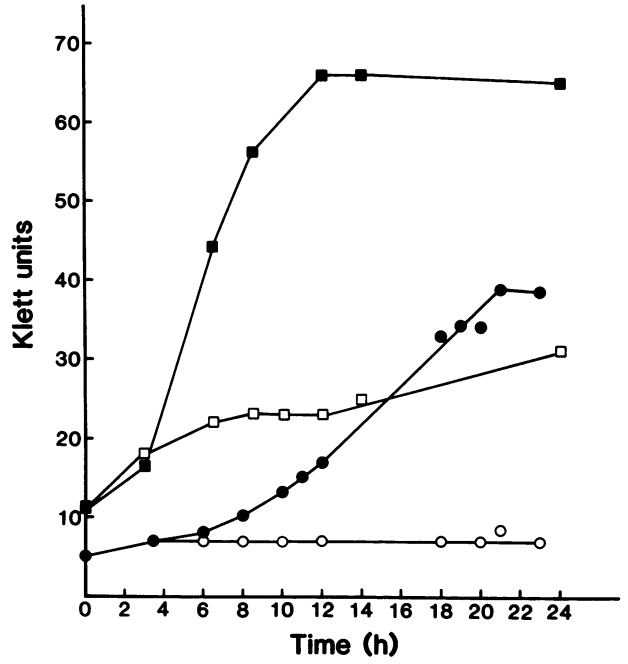

FIG. 1. Anaerobic growth of PL2024 on glycerol minimal and glycerol complex medium in the presence and absence of THTO. Symbols: ○, minimal medium; ●, minimal medium plus THTO; □, complex medium; ■, complex medium plus THTO.

The contents of the tubes were incubated at 30°C for 30 min for natural donors and for 5 min for artificial donors. The reaction was terminated by the addition of 4 drops of 5 N HCl, the mixture was cooled in an ice bath, 1 ml of a saturated solution of NaCl was added, and the mixture was centrifuged at 14,000 × *g* for 10 min to remove the protein. The supernatant was extracted with 2 ml of methylene chloride, and the layers were separated by centrifugation for 5 min in a clinical centrifuge. Samples of the methylene chloride layer were used for gas chromatography. Control experiments without an electron donor were run, and the small amount of endogenous activity obtained was always subtracted.

The quantity of the reaction product, THT, was determined by gas chromatography. A glass column (6 ft [ca. 1.8 m] by 2 mm or 6 ft by 4 mm) containing Carbopack B–1.5% XE60–1% H_3PO_4 was used. The instrument used was a Packard model 437 gas chromatograph. It was operated isothermally at 135°C, with the flame ionization detector and injector at 140 and 135°C, respectively. Standards containing known quantities of THT were always tested along with the samples. The quantities of THT in the experimental samples were calculated by comparing the peak areas of known standards with those of the samples.

Growth of *E. coli* strains on glycerol complex medium containing THTO. Wild-type *E. coli* PL2024 failed to grow in glycerol minimal medium anaerobically in the absence of THTO. In the presence of THTO, however, growth occurred after a long lag; in glycerol complex medium, increased growth and a decreased lag period were observed (Fig. 1).

The *chlA*, *chlB*, *chlD*, and *chlE* mutants were unable to grow on glycerol complex medium containing THTO (Table 2). However, supplementation of the medium with 10 mM sodium molybdate allowed full growth of the *chlD* mutant.

TABLE 2. Growth of *chl* and *men* mutants in the presence and absence of various supplements[a]

Strain	Growth with the following supplement[b]:			
	None	Molybdate	OSB	DHNA
chlA	20	18	—[c]	—
chlB	19	21	—	—
chlD	15	62	—	—
chlE	13	21	—	—
menB	14	—	21	56
menC	28	—	76	66
menD	12	—	65	62

[a] Strains were inoculated into glycerol-THTO medium in the presence and absence of various supplements and grown anaerobically.
[b] Growth was monitored with a Klett meter, and the maximum growth attained is shown. Sodium molybdate was added at a concentration of 10 mM; OSB and DHNA were added at 10 and 4 μM, respectively.
[c] —, Not applicable.

Mutants of *E. coli* that are blocked in the menaquinone biosynthetic pathway, i.e., *menB*, *menC*, and *menD* mutants, were unable to grow on glycerol complex medium containing THTO (Table 2). Addition of either of the menaquinone biosynthetic intermediates, OSB or DHNA, to the growth medium restored full growth of *menC* and *menD* mutants. In contrast, OSB had no effect on the growth of the *menB* mutant, whereas DHNA restored growth (Table 2).

Growth conditions and THTO reductase activity. Cell extracts of wild-type *E. coli* PL2024 were assayed for reduction of THTO to THT with various electron donors (Table 3). In decreasing order of efficiency, reduced benzyl viologen (BVH_2), dithionite, NADH, formate, NADPH, and lactate functioned as electron donors. The enzyme activities in cells grown anaerobically on glucose and on glucose plus THTO were approximately equal with all the natural donors under all the growth conditions (Table 3). In assays with BVH_2 or dithionite, the activities were higher in glycerol-THTO-grown cells.

To determine whether the enzymes are membrane bound, the 3,000 × *g* supernatant from glycerol-THTO-grown cells was further centrifuged at 150,000 × *g*. The enzyme activities assayed with NADH, formate, BVH_2, and dithionite were much higher in the pellet than in the supernatant fraction (Table 3).

Enzyme activity in the *chlD* and *men* mutants. Although,

TABLE 3. Effect of growth conditions and ultracentrifugation on enzyme activity[a]

Electron donor	Enzyme activity (nmol/min per mg of protein) with the following growth conditions:				
	Glucose (3,000 × *g* supernatant)	Glucose THTO (3,000 × *g* supernatant)	Glycerol THTO (3,000 × *g* supernatant)	Glycerol-THTO (150,000 × *g*)	
				Supernatant	Pellet
NADH	5.9	9.5	10.7	2.5	44
Formate	9.0	7.2	7.7	0.5	23
NADPH	ND[b]	3.5	5.7	1.3	1.5
Lactate	2.3	1.4	3.6	0.7	3.3
BVH_2	110	100	170	110	460
Dithionite	37	36	65	50	210

[a] The cells were grown anaerobically in the various media, and cell extracts were assayed.
[b] ND, Not determined.

TABLE 4. Enzyme activity in *chlD* and *men* mutants grown in the presence and absence of growth supplements[a]

Strain	Supplement	Enzyme activity (nmol/min per mg of protein) with:	
		NADH	BVH_2
PL2024	Molybdate	8	119
chlD	None	0.3	18
	Molybdate	20	109
menB	None	0.8	120
	OSB	0.7	95
	DHNA	5.5	70
menC	None	0.05	88
	OSB	6.8	95
	DHNA	3.5	90

[a] The cells were grown in glucose-THTO medium anaerobically in the presence and absence of the indicated supplements.

the *chlD* mutant failed to grow on glycerol-THTO medium (Table 2), it could be grown on glucose-THTO. The THTO reductase activity in such cells was very low. However, growth of the mutant in the presence of molybdate restored the enzyme activity (Table 4). Growth of the wild-type strain in the presence of molydbate did not change the level of enzyme activity. Compared with the wild-type strain, *chlD* cells grown in the presence of molybdate showed an unusually high level of activity when NADH was used as electron donor. This observation is attributed to strain differences. This work establishes a role for a molybdenum-containing cofactor in the THTO reductase system.

The *men* mutants did not grow on glycerol-THTO medium unless a menaquinone intermediate was present (Table 2), but they could be grown fermentatively on a glucose medium. Hence, cell extracts of a *menB* mutant (JRG962) and a *menC* mutant (JRG862) grown in glucose medium were assayed for enzyme activity (Table 4). The enzyme activity was low when assayed with NADH, NADPH, formate, and lactate, but remained the same as in the glucose-grown wild-type strain when assayed with BVH_2 and dithionite (only the data for NADH and BVH_2 are shown). As shown above, growth of the *menC* mutant could be restored by the addition of either OSB or DHNA to the glycerol-THTO medium; with the *menB* mutant, only DHNA restored growth (Table 2). Consistent with this observation, when the *menC* mutant was grown in the presence of OSB or DHNA, full enzymatic activity was restored. OSB had no effect on the enzyme activity in the case of the *menB* mutant, but DHNA restored activity (Table 4). These observations are in accord with the known metabolic blocks in the pathway for menaquinone biosynthesis (3).

The results reported here show that *E. coli* reduces THTO to THT by using NADH, NADPH, formate, lactate, BVH_2, or dithionite as the electron donor. The reductase responsible for the conversion of THTO to THT and the dehydrogenases linking the electron donors NADH and formate are membrane bound. The activities of the various dehydrogenases are about the same in cells grown with glucose or glycerol as the carbon source. However, the activity of the reductase from glycerol-grown cells assayed with BVH_2 or dithionite was approximately twice that of reductase from glucose-grown cells.

With the naturally occurring electron donors, cell extracts of the *men* mutants showed low levels of THTO reduction. However, with BVH_2 and dithionite, the activities were similar to those of the wild type. The results with the *men* mutants show that menaquinone is involved in the transfer of electrons from NADH, NADPH, formate, and lactate, but not in the reduction of THTO to THT. When dithionite or BVH_2 is used, the electrons are probably transferred directly to THTO without the involvement of the electron transport chain. The functions of menaquinone have been reviewed by Taber (17). These studies demonstrate that THTO reduction, like fumarate and TMAO reduction, is menaquinone dependent.

The THTO reductase system seems to loosely resemble TMAO and DMSO reduction. However, drawing an analogy between the three systems becomes complicated by the fact that there are three or four inducible and one constitutive TMAO reductase (2). In a glucose-containing medium, TMAO increases growth of *E. coli* by two- to threefold (7). In contrast, with DMSO and THTO, *E. coli* showed only slight improvement in growth (data not shown). A further complication is that in the presence of TMAO, the pH of the medium increases, thus possibly neutralizing the acids produced during fermentation. It was reported by Bilous and Weiner (4) that 77% of DMSO reductase was associated with the membrane, while only 53% of TMAO reductase was membrane bound. Our studies show that under similar conditions, about 80% of THTO reductase is membrane bound. Although 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide is not an inhibitor of TMAO reduction (6), DMSO reduction is highly sensitive to this material (5). Further, TMAO reductase activity in extracts of glucose-TMAO-grown cells is about 15-fold higher than in extracts of glucose-grown cells (18), and extracts of fructose-TMAO-grown cells contain 30-fold-greater TMAO reductase activity compared with fructose-grown cells (8). In contrast, our results show that the THTO reductase activity remains unchanged when extracts from glucose-THTO-grown cells are compared with those from glucose-grown cells. Further, it has been reported that DMSO reductase is an inducible enzyme. When the specific activity of DMSO reductase from cells grown aerobically on glycerol-DMSO medium was compared with that from cells grown anaerobically on the same medium, there was a 55-fold increase in activity (4). In contrast, under similar conditions, THTO reductase activity increased by only 2.5-fold. However, it is possible that one or more of the TMAO reductases act on DMSO and THTO. Further studies are required to establish whether the reductions of TMAO, THTO, and DMSO are carried out by the same or different enzymes.

We thank Barbara Bachmann and J. R. Guest for the strains of *E. coli*.

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