A NOVEL GENE, *torB*, FOR TRIMETHYLAMINE N-OXIDE REDUCTASE IN *ESCHERICHIA COLI*

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Trimethylamine *N*-oxide (TMAO) reductase which is involved in anaerobic respiration is one of the molybdenum-containing enzymes in *Escherichia coli*. A mutant defective in the activity of TMAO reductase was isolated by inserting phage *MudI*. The mutation was localized at 0.7 min adjacent to *chlG* on the *E. coli* chromosome, and was designated *torB*. The *torB* mutant grown anaerobically with TMAO contained 10–30% of the enzyme activity in the parent strain grown under the same condition and about 80% of the enzyme protein immuno-reactive with an antiserum against the TMAO reductase. The content of molybdopterin, an organic moiety of molybdenum cofactor, in the immuno-precipitate from extracts of the *torB* mutant was rather low, about 60% of that in the immuno-precipitate from the parent strain. The mutant showed a normal level of another molybdenum enzyme nitrate reductase activity when anaerobically grown with nitrate. These findings suggest that the *torB* gene is involved in the formation of the active enzyme of TMAO reductase through the processing of molybdenum cofactor. There was a marked expression of *torB* under anaerobic conditions, and TMAO was not required for it.

Some bacteria can use trimethylamine *N*-oxide (TMAO) as a terminal electron acceptor under anaerobic conditions (3). In *Escherichia coli*, the electron transport system of TMAO reduction is composed of some dehydrogenases, cytochromes, quinones, and TMAO reductase. This bacterium has three to four forms of TMAO reductase which are electrophoretically distinguishable (27). TMAO reductase I and III are inducible by TMAO and are composed of two identical peptides and a single one, respectively (28; Yamamoto and Hinakura, unpublished). TMAO reductase

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Abbreviations used in this text are: TMAO, trimethylamine *N*-oxide; DMSO, dimethyl sulfoxide.

II is rarely detected, and TMAO reductase IV is a constitutive enzyme. The latter is probably the same one that Weiner et al. (33) recently named dimethyl sulfoxide (DMSO) reductase. An antiserum prepared against TMAO reductase I reacts with reductases I and III, but not with reductase IV (28; Yamamoto and Hinakura, unpublished).

TMAO reductase I (35) was shown to contain molybdenum and molybdopterin as components of the molybdenum cofactor. The synthesis, processing, and insertion of molybdenum cofactor involve several genes *chlA*, *chlB*, *chlD*, *chlE*, and *chlG* (22), and in *E. coli* the *chl* mutations cause a marked decrease in the activities of some molybdenum-containing enzymes including nitrate reductase (25), formate dehydrogenase (25), biotin sulfoxide reductase (6), and DMSO reductase (4). TMAO reductase is less active in the *chlA*, *chlB*, *chlD*, and *chlE* mutants of *E. coli*, but the *chlG* mutant strain HEJ1 shows high activity, as does a wild-type strain (32). The *fnr* gene product is a positive regulator for the expression of genes for some anaerobic enzymes including nitrate reductase and fumarate reductase (8, 26). The mutation of the *fnr* gene causes a great loss of TMAO reductase activity (28, 30), but the synthesis of the enzyme protein is not regulated by the *fnr* gene product (24, 28). Recently, two genes which specifically govern TMAO reductase have been found in *E. coli*. One of them, named *tor*, which lies between 66 min and 85 min on the *E. coli* chromosome (31), is a regulatory gene (Hohmura et al., unpublished). The other is the structural gene *torA* which is located at 28 min (24). A *tor* mutation has also been found at 83 min in *Salmonella typhimurium* (19).

To investigate a mechanism for genetic regulation of the TMAO reductase, we isolated mutants defective in the reductase activity, other than the chlorate-resistant mutants, by the *MudI* insertion technique of Casadaban and Cohen (7). Among the *MudI* lysogens, one strain, which showed a low activity of TMAO reductase, had a mutation in a gene different from *torA* and *tor*. In this study we describe the biochemical and genetic properties of the new gene, designated *torB*.

MATERIALS AND METHODS

Bacterial strains and phages. The *E. coli* strains used in this study are listed in Table 1. *MudI* phage was prepared from the *E. coli* strain MAL103. P1 phage was multiplied in the *E. coli* strain HM168.

Mutant isolation. To isolate mutants defective in TMAO reductase activity, the *MudI* phage insertion method was used according to Casadaban and Cohen (7). Strain AB2277 was infected with *MudI*, and the cells were plated on MacConkey agar (BBL) containing 40 mM TMAO, 0.2% glucose, and 0.01% ampicillin (MTGA plate), as described by Davidson et al. (10). Red colonies were preliminarily identified as TMAO reductase-defective mutants.

Genetic analysis. Experiments with Hfr conjugation were carried out basically according to Miller (23). After mating the mutant and Hfr strain with incubation

Table 1. *E. coli* strains used in this study.

Strain	Genotype	Source or reference
MAL103	F ⁻ <i>araD139 lacU169 rpsL thi ara::MudI</i> (Ap ^r <i>lac</i>)	M. Casadaban (7)
AB2277	F ⁻ <i>ilv met his trp pro thi lac mal ara gal str</i>	E. A. Adelberg
HM10	AB2277 with <i>torB::MudI</i> (Ap ^r <i>lac</i>)	this study
HM168	thermo-stabilized HM10	this study
Hfr3000	Hfr <i>thi1 relA1 spoT1 supQ80</i>	CGSC259
K10	Hfr <i>rel1 tonA22</i>	CGSC4311
LCB320	F ⁻ <i>thr1 leuB6 lacY1 tonA21 str</i>	M. Chippaux (5)
JF1130	F ⁻ <i>leuB6 proA2 his4 argE3 thi pyrE60 lacY galK2 xyl15 mtl1 ara14 chlG6 rpsL31 supE44</i>	CGSC5567
TR111	F ⁻ <i>tor argE thi1 rpsL</i>	M. Takagi (31)
LCB128	$\Delta(lac)$ U169 <i>rpsL torA::MudI</i>	M.-C. Pascal (24)

CGSC: *E. coli* Genetic Stock Center, Yale University School of Medicine, New Haven, Connecticut, U.S.A.

for 50 min, recombinants were selected on appropriate plates containing streptomycin, 50 μ g/ml, and scored for ampicillin sensitivity on plates containing 0.01% ampicillin. P1 transduction was performed as described (23). Approximate map distances were determined by the formula of Wu (34).

Preparation of cell extracts. Cells were grown in LB broth (23) at 37°C under anaerobic conditions established by bubbling with N₂. When indicated, 50 mM TMAO or 50 mM KNO₃ was added. Optical density at 660 nm was measured in a 10 mm-light path cuvette with a spectrophotometer (Jasco uvidec 340).

To prepare cell-free extracts, 8 h incubation cultures were harvested by centrifugation at 10,000 $\times g$ for 10 min. Cells suspended in 50 mM Tris-HCl, pH 7.4, were disrupted with a sonicator (Tomy UR-150P) at 20 kHz and 74 W for 5 min then centrifuged at 10,000 $\times g$ for 10 min. The supernatants were used as crude extracts. When indicated, the crude extracts were ultracentrifuged at 100,000 $\times g$ for 90 min. The obtained supernatant was used as a soluble protein fraction (S₁₀₀). The precipitate was solubilized with 1% sodium *N*-lauroyl sarcosinate in the buffer at room temperature then ultracentrifuged again. The supernatant obtained was used as a membrane protein fraction (P₁₀₀).

Assays of TMAO reductase and nitrate reductase. The activities of TMAO reductase (35) and nitrate reductase (36) were determined by monitoring the absorbance of dithionite at 315 nm and 30°C in a 1 mm-light path cuvette; a 9 $\times$ 9 mm quartz spacer was inserted into a 10 $\times$ 10 mm cuvette. TMAO reductase activity was assayed in a reaction mixture (0.6 ml) containing 50 mM acetate buffer, pH 5.5, 2 mM benzyl viologen, 15 mM TMAO, 3.3 mM Na₂S₂O₄, and 5 mM KHCO₃. To determine nitrate reductase activity, the reaction mixture was composed of 50 mM Tris-maleate, pH 6.9, 15 mM KNO₃, 1 mM benzyl viologen, 3.3 mM Na₂S₂O₄, and 5 mM KHCO₃. One unit (U) of either enzyme is defined as the amount that catalyzes a decrease of 1 μ mol of dithionite per min.

Protein was determined by the method of Lowry et al. (21) with bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis. The electrophoresis was carried out according to Davis (11) with 7.5% disc gels. Gels were stained for TMAO reductase activity as described before (27).

Immunological methods. An antiserum against the TMAO reductase was obtained by immunizing male rabbits with the purified TMAO reductase I (Yamamoto and Okubo, unpublished). Rocket immunoelectrophoresis was carried out in an electrophoresis chamber (Bio-Rad 1415) with 1% agarose gel containing 0.8% of the antiserum on glass plates at 2.5 mA per 12 cm of gel width at 4°C. The purified TMAO reductase I was used for calibration. Antibody reaction was tested using the Ouchterlony double immunodiffusion method (17).

Assay of molybdenum cofactor. Amounts of molybdopterin was determined by measuring fluorescence spectra in immuno-precipitates. The S_{100} fractions and the antiserum were mixed and incubated at 20°C for more than 5 h. The immuno-precipitates formed were collected by centrifugation, washed with saline, then suspended in 10 mM Tris-HCl, pH 7.4. The form A derivative of molybdopterin was prepared from the immuno-precipitates as described previously (16, 35), except omitting the chromatographies. The fluorescence spectrum was recorded with excitation at 385 nm.

*Assay of *torB-lacZ* gene fusion expression.* To assay *torB-lacZ* fusion expression, β -galactosidase activity was measured by the *o*-nitrophenyl galactoside procedure and expressed in Miller units (23). Cells growing in LB broth at 37°C were harvested at a middle logarithmic phase; Aerobically grown cells were obtained by shaking 10 ml of culture in a 100 ml flask at 102 strokes/min for 3 h, and anaerobic cultures were obtained by incubation for 7 h in a jar with GasPak system (BBL). Cells suspended in Z buffer were permeabilized with chloroform and sodium dodecyl sulfate.

RESULTS

Isolation of a TMAO reductase-defective mutant

Strain AB2277 was infected with MudI phage and lysogens were selected at 27°C on MTGA plates as red ampicillin-resistant colonies (37 of 13,884 lysogens). These colonies were transferred to a nutrient agar containing 0.1% KClO₃ and anaerobically incubated for 2 days. Twenty strains among 37 strains grew well and were discarded as chlorate resistant mutants. The remaining 17 strains were tested for TMAO reductase activity in crude extracts of the cells. Among these strains grown anaerobically with TMAO, only one strain (HM10) showed a low activity of TMAO reductase, 0.22 U/mg protein, about 10% of that in the parent strain AB2277. After incubating strain HM10 at 42°C, a red ampicillin-resistant colony was isolated as a MudI-stabilized strain (HM168). This strain also had very low TMAO reductase activity (Table 2). When strains HM10 and HM168 were grown

Table 2. Activities of nitrate reductase and TMAO reductase in crude extracts prepared from *E. coli* strains grown anaerobically on LB broth with and without electron acceptors.

Strain	Activities (U/mg protein)			
	Nitrate reductase		TMAO reductase	
	–NO ₃ [–]	+NO ₃ [–] ^a	–TMAO	+TMAO ^a
AB2277	0.233	1.50	0.241	2.01
HM168 (<i>torB</i>)	0.115	1.54	0.041	0.635
JF1130 (<i>chlG</i>)	0.137	0.352	0.259	1.46

^a The concentrations were 50 mM in the medium.

on lactose-containing MacConkey agar plates, colonies of HM168 showed red, but those of HM10 were white. This suggests that strain HM168 contains a gene fusion with *lacZ*. Strain HM168 was used for further characterization of the mutant.

When strain HM168 was anaerobically grown in LB broth at 37°C, the doubling time of growth (87 min) was the same in the absence or presence of 50 mM TMAO or 50 mM nitrate during 0–3 h incubation, while for the parent strain AB2277 it was 43 min with nitrate and 55 min with or without TMAO.

Genetic mapping

Strain HM168 was conjugated with some kinds of Hfr strains to map the position of TMAO reductase-defective mutation. When the conjugates from the mating with strain Hfr3000 (clockwise transfer, origin at 97 min) were isolated on proline-less plates with streptomycin, they were susceptible to ampicillin (Ap^s) at a ratio of 61% (63/104). Conjugates obtained on similar plates by mating with strain K10 (counterclockwise transfer, origin at 13 min) showed Ap^s at 9% (9/104). Thus the mutation was located in the region containing *thr* between the transfer origin of strain Hfr3000 (97 min) and *pro* (6 min) in the linkage map of *E. coli* (2). This region is clearly distinct from the *tor* locus (66–85 min, 31) and from the position of the structural gene *torA* (28 min, 24). The new gene was designated *torB*.

Transduction with P1 phage propagated in strain HM168 was carried out to locate the mutation (Table 3). Transductants of LCB320 to *thr*⁺ showed ampicillin resistance (Ap^r) at a ratio of 30%. Lac⁺ due to expression of *torB-lacZ* fusion was also observed at 33% (49/176). Of the *thr*⁺ Ap^r transductants, 7% (9/133) were co-transductants with the *ara* gene. Transductants isolated as *leu*⁺ did not show Ap^r. Though isolation of the *thr*⁺ *leu*⁺ double transductants from LCB320 was tried, no transductant was obtained. Of the Ap^r transductants of the *chlG* mutant JF1130, 86% were chlorate-sensitive cells, which showed high activities of nitrate reduction (data not shown). One of transductants isolated as *leu*⁺ from strain JF1130 showed Ap^r, but such a strain could not be obtained again in other experiments. These results indicate that *torB* is located at 0.7 min between *thr* and *ara* loci,

Table 3. Mapping by P1 transduction of *torB*. Ap^r due to *MudI* (Ap^r *lac*) was adopted as a marker for *torB* gene.

Donor	Recipient	Selected marker	Unselected marker	No. of transductants tested	No. of co-transductants	% of co-transduction
HM168	LCB320	<i>thr</i> ⁺	Ap ^r	438	133	30
			<i>ara</i> ⁻	449	36	8
		<i>leu</i> ⁺	Ap ^r	469	0	0
	JF1130		<i>ara</i> ⁻	365	236	65
		<i>leu</i> ⁺	Ap ^r	458	1	0.2
			Chl ^s	399	0	0
		Ap ^r	Chl ^s	22	19	86

Table 4. Specific activity of TMAO reductase and content of protein reactive with the antiserum against TMAO reductase I in *E. coli* strains grown anaerobically in the presence of 50 mM TMAO.

Strain	TMAO reductase activity (U/mg of protein)		Immuno-reactive protein (μg/mg of protein)	
	S ₁₀₀	P ₁₀₀	S ₁₀₀	P ₁₀₀
AB2277	3.49	3.97	17.2	36.5
JF1130 (<i>chlG</i>)	2.56	4.79	16.2	37.3
HM168 (<i>torB</i>)	0.425	0.841	13.8	31.6

although the orientation with respect to *chlG* could not be determined in this experiment.

Biochemical characterization of *torB* mutant

Activities of TMAO reductase and nitrate reductase were determined in extracts prepared from the *torB* strain HM168, its parent strain AB2277, and a *chlG* mutant strain JF1130 (Table 2). In strain HM168, when cells were anaerobically grown on LB broth in the presence of 50 mM nitrate, nitrate reductase was induced; the specific activity was equal to that in strain AB2277. Under anaerobic growth conditions with TMAO, strain HM168 showed a specific activity of TMAO reductase that was 20–30% of that in strain AB2277. With gel staining for TMAO reductase activity after polyacrylamide gel electrophoresis of the crude extract, bands of TMAO reductases I, III, and IV in the mutant were faintly visualized over 30 min of incubation in the staining solution, while these bands of the enzyme in the parent strain appeared within 5 min. TMAO reductase II was not found in any extracts.

Protein immuno-reactive with a specific antiserum against TMAO reductase I appeared in S₁₀₀ and P₁₀₀ fractions (Table 4). In both fractions from strain HM168, the contents of the enzyme protein were 70–90% of those from the parent strain AB2277. A single immuno-precipitin band without a spur was formed between the S₁₀₀ fractions prepared from strains HM168 and AB2277 in the Ouchterlony

Fig. 1. Fluorescence spectra of the form A derivative of molybdenum cofactor in immuno-reactive proteins with the antiserum against TMAO reductase I. Immuno-precipitates were prepared from S_{100} fractions of following strains: A, strain AB2277 (0.28 mg protein of precipitate); B, strain HM168 (0.31 mg). Preparation of the molybdenum cofactor derivative was obtained as described in MATERIALS AND METHODS. Fluorescence spectra were obtained with excitation at 385 nm.

double immunodiffusion test. Crude extracts of a *tor* mutant TR111 and a *torA* mutant LCB128 grown anaerobically with 50 mM TMAO showed very low TMAO reductase activity (about 0.25 U/mg protein) and contained little TMAO reductase protein (less than 0.5 μ g/mg of protein).

Form A derivative of molybdopterin, an organic component of molybdenum cofactor, was prepared from immuno-precipitates from S_{100} fractions and fluorescence spectra were measured (Fig. 1). Peak heights of spectra per mg of immuno-precipitate protein showed that the content of molybdopterin in the protein of the TMAO reductase of strain HM168 was only 55–64% of that in the strain AB2277 enzyme.

Strain HM168 anaerobically grown in the absence of TMAO showed 20% of the TMAO reductase activity in the parent strain (Table 2). The *torB* gene mutation seems to affect the activity of the constitutive enzyme TMAO reductase IV.

In the *chlG* mutant strain JF1130, induction of nitrate reductase by nitrate was very low, but levels of the activity and immuno-reactive protein of TMAO reductase were almost equal to those in strain AB2277.

Response of torB expression to growth conditions

The *torB* gene was shown above to be fused with *lacZ* on *MudI* in strain HM168. To determine the degree of expression of *torB-lacZ* gene fusion, β -galactosidase activity was determined in the cells grown under a variety of conditions. The results are shown in Table 5. High activities of β -galactosidase were observed under anaerobic growth conditions compared with the activity in aerobic cultures. Adding TMAO did not affect the level of the activity. Neither nitrate, molybdate, nor tungstate had much effect on the expression of *torB*.

Table 5. Expression of *torB-lacZ* fusion. The strain HM168 grown on LB broth containing ampicillin (30 μ g/ml) was used to assay β -galactosidase activity.

Growth condition	Additives	β -Galactosidase activity (Miller units)
Aerobic	None	224
	40 mM TMAO	207
Anaerobic	None	839
	40 mM TMAO	934
	40 mM KNO ₃	760
	40 mM TMAO + 40 mM KNO ₃	808
	1 mM Na ₂ MoO ₄	845
	40 mM TMAO + 1 mM Na ₂ MoO ₄	1,090
	1 mM Na ₂ WO ₄	786
	1 mM Na ₂ WO ₄ + 1 mM Na ₂ MoO ₄	830

DISCUSSION

In this study we isolated a TMAO reductase-defective mutant by using the MudI phage insertion method. The *E. coli* TMAO reductase is a molybdoprotein containing molybdenum and molybdopterin which is the same organic moiety of the molybdenum cofactor as that in nitrate reductase (1, 14, 35). Because the *chlA*, *chlB*, *chlD*, *chlE* and *chlG* genes are involved in molybdenum cofactor synthesis, insertion or molybdate uptake, chlorate-resistant mutants were excluded in the course of isolation. The *torB* mutation obtained in this way was shown to be localized at 0.7 min on the *E. coli* chromosome map, indicating the location of *torB* is clearly distinct from that of known genes *tor* (between 66–85 min, 31) and *torA* (at 28 min, 24).

The *torB* mutant showed a low activity of TMAO reductase and contained 70–90% of the enzyme protein compared with that in the parent strain. The content of molybdenum cofactor in the mutant enzyme was about 60% of that in the wild-type enzyme. Though these findings are insufficient to deduce a function of a *torB* gene product, *torB* seems to be involved in insertion or processing of molybdenum cofactor for TMAO reductase, but it neither encodes the enzyme protein nor regulates the structural gene.

The *chlG* mutant showed normal levels of TMAO reductase activity when grown anaerobically with TMAO (32, Table 2), though *chlA*, *chlB*, *chlD*, and *chlE* are required to produce enzymatically active TMAO reductase (32). The *chlG* mutation has no effect on the activity of formate dehydrogenase-N, a molybdenum-containing enzyme in *E. coli* (12). In contrast, nitrate reductase requires a *chlG* gene product for the enzyme activity, as shown in Table 2. The function of the *chlG* gene apparently inserts molybdenum into a demolybdo form of the cofactor (30). If so, molybdenum cofactors in the respective enzymes should

have partly different structures. In fact, there is a difference in the chemical bonds of molybdenum in the cofactors from two chicken enzymes sulfite oxidase and xanthine dehydrogenase (9). These findings indicate that *E. coli* has at least a few genes involved in the processing of molybdenum cofactors corresponding to the respective enzymes. The *torB* is one such gene which specifically functions for TMAO reductase.

TMAO reductase activity has been shown to be low in cells of *E. coli* grown under aerobic conditions or anaerobic conditions with nitrate or tungstate (13, 27). *MudI* insertion frequently results in a transcriptable gene fusion with *lacZ* on the *MudI* genome, and such a gene fusion is useful to determine the degree of the gene expression. In this study the *torB* gene was expressed preferably in anaerobic cultures. Neither stimulation nor inhibition of the *torB* expression was observed in the presence of TMAO, nitrate, molybdate, or tungstate. Pascal et al. (24) reported that the expression of *torA-lacZ* gene fusion is anaerobically induced by TMAO and repressed under aerobic conditions. The synthesis of TMAO reductase protein is not repressed by adding nitrate or tungstate to *E. coli* cultures, but the enzyme activity is decreased under these growth conditions (28). These observations indicate that the transcription of each gene *torA*, *torB*, and *tor* is not controlled by nitrate and tungstate. The negative effects of these compounds appear to be due to competition of molybdenum cofactor incorporation between TMAO reductase and nitrate reductase induced by nitrate and production of metal-free or tungsten-containing cofactor.

The *fnr* gene product positively controls the expression of the *nar* (8, 20, 29) and *frd* (15, 18, 20) operons. Though *fnr* mutation does not affect the expression of the structural gene *torA*, it results in a decrease in TMAO reductase activity (24, 28). This phenomenon is similar to the *torB* mutation with respect to the TMAO reductase activity, indicating that the *fnr* product may regulate the transcription of *torB*.

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