Short Communication

EFFECT OF THE *tor* MUTATION IN *ESCHERICHIA COLI* ON THE TRIMETHYLAMINE *N*-OXIDE, NITRATE AND DIMETHYLSULFOXIDE REDUCTASES, THE FORMATE DEHYDROGENASES N AND H AND NITRATE REPRESSION

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In *Escherichia coli*, trimethylamine *N*-oxide (TMAO) reductase is one of the anaerobic respiratory enzymes like nitrate reductase and fumarate reductase (1, 11, 26). Takagi and Ishimoto (30) have isolated a *tor* mutant of *E. coli* which is defective in TMAO reductase activity but shows nitrate reductase activity in intact cells. This mutant, strain TR111, does not contain an immunologically detectable protein of TMAO reductase (35). However, we recently found that nitrate reductase activity was low in cell-free extracts prepared from the mutant. Since this predicts that the *tor* mutation is pleiotropic, effects of the mutation on several enzymes involved in anaerobic respiration were investigated in this study. A locus of the mutation was also mapped on the *E. coli* chromosome.

E. coli strains used were: AN180 (F⁻ *thi argE* Str^r), TR111 (as AN180 but *tor*; (30)), KL228 (Hfr *thi-1 leuB6 gal-6 lacY1* or *lacZ4 supE44*; CGSC4318), RR1 (F⁻ *proA2 lacY1 hsdS20 rpsL20 ara-14 galK2 xyl-5 mtl-1 supE44*), IM313 (*bgl nalA* derivative of TR111; this study) HM176 (Hfr *tor thi-1 bgl nalA*; this study), HM177 (F⁻ *tor thi-1 hsdS20 rpsL20 lacY leuB6*; this study), and JF1130 (F⁻ *thi-leuB6 proA2 his-4 argE3 pyrE60 lacY1 galK2 xyl-5 mtl-1 ara-14 chlG6 rpsL31 supE44*; CGSC5567). IM313 was isolated according to Miller (20). HM176 was

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Abbreviations: TMAO, trimethylamine *N*-oxide; DMSO, dimethyl sulfoxide.

constructed by mating IM313 with KL228. HM177 was obtained by conjugation between strains HM176 and RR1.

Cells were anaerobically grown at 37°C for 12 h in LB medium (20) which filled the neck of a flask. When indicated, concentrations of TMAO and KNO₃ in the medium were 50 mM. Cell-free extracts were prepared as described (34). Protein was determined by the Lowry method.

Enzyme activities were assayed at 30°C with a spectrophotometer (Jasco Uvidec 340). Reductase activities were determined by monitoring the decrease in dithionite ($\epsilon = 8 \text{ mM}^{-1} \text{ cm}^{-1}$ at 315 nm) used as an electron donor, as described previously for TMAO reductase assay (34). Reaction mixtures used were according to; Yamamoto et al. (34) for TMAO reductase, Enoch and Lester (9) for nitrate reductase, Weiner et al. (31) for dimethyl sulfoxide (DMSO) reductase, and Lemier et al. (17) for fumarate reductase.

The activities of the nitrate reductase- and hydrogenase-linked formate dehydrogenases were determined by the Lester and DeMoss methods (18), except that 3-(4,5-dimethyl thiazolyl-2-) 2,5-diphenyltetrazolium bromide (for its formazan $\epsilon = 17 \text{ mM}^{-1} \text{ cm}^{-1}$ at 570 nm) was used instead of DCPIP to assay the activity of nitrate reductase-linked formate dehydrogenase N.

Hydrogenase activity was determined by monitoring the reduction of methylene blue ($\epsilon = 28 \text{ mM}^{-1} \text{ cm}^{-1}$ at 668 nm) with hydrogen saturated in a 50 mM phosphate buffer, pH 7.0, containing 72 μM methylene blue in a rubber-sealed cuvette.

Conjugation experiments with different Hfr strains have suggested that the *tor* mutation in strain TR111 lies in the 66–85 min region (30). A *tor* gene also has been found between 80 and 83 U near *xyl* on the *Salmonella typhimurium* chromosome (15). The *tor* in *E. coli* was mapped by transduction with P1 phage according to Miller (20). Transductants of *tor*⁻ were identified as red colonies after 8 h incubation at 37°C on MTG plates composed of MacConkey agar (BBL), 40 mM TMAO, and 0.2% glucose as described (15, 30). P1 was propagated in strain TR111, and strain JF1130 (*xyl* [79.7 min], *mtl* [80.7 min]) was used as a recipient. Transductants isolated as *xyl*⁺ showed *tor*⁻ at a ratio of 44.3% (77/174). Of the *mtl*⁺ transductants, 56.8% (67/118) were co-transductants with *tor*⁻. The *xyl*⁺ *mtl*⁺ double transductants showed *tor*⁻ at a high ratio (39/40), indicating that *tor* mutation is located between *xyl* and *mtl*. This locus is clearly distinct from *torA*, the structure gene of TMAO reductase (28 min; 23), and *torB* involved in the insertion or processing of molybdenum cofactor for TMAO reductase (0.7 min; 35). We propose the term of *torR* mutation instead of *tor* since its effect is pleiotropic as shown below.

Three strains, the *torR* (originally named *tor*) strain TR111, its parent strain AN180, and a strain HM177 (*torR*⁻ recombinant constructed by mating) were grown anaerobically with and without electron acceptors, and the activities of some anaerobic respiratory enzymes were determined in cell-free extracts (Table 1). In strain AN180, induction of TMAO reductase by TMAO and nitrate reductase by nitrate were observed as well as nitrate reductase-linked formate dehydrogenase N.

Table 1. Enzyme activities in cell-free extracts prepared from the wild type strain AN180, the *torR* mutant TR111, and strain HM177 which was isolated as a *torR* strain by conjugation. The strains were grown anaerobically with or without 50 mM electron acceptors.

Strain	Grown with	Enzyme activities (nmol/min/mg of protein)						
		TMAO reductase	Nitrate reductase	DMSO reductase	Formate dehydrogenase N	Formate dehydrogenase H	Fumarate reductase	Hydrogenase
AN180	None	988	430	145	41.3	10.1	624	37.6
	TMAO	4,130	264	170	133	8.9	1,950	52.0
	Nitrate	73.2	2,220	9.8	102	0.0	9.8	0.0
TR111	None	72.4	151	19.7	0.7	0.0	776	39.0
	TMAO	311	121	21.1	1.6	0.0	1,040	42.7
	Nitrate	66.3	151	6.0	0.6	0.0	404	17.2
HM177	None	58.0	177	8.3	0.6	2.4	601	43.3
	TMAO	181	107	3.8	0.5	0.0	407	28.4
	Nitrate	40.0	204	1.7	0.6	0.0	846	27.8

There were also activities of DMSO reductase, formate dehydrogenase H, fumarate reductase, and hydrogenase in the cells grown without electron acceptors. Also, nitrate repressed DMSO reductase (2), formate dehydrogenase H (18), fumarate reductase (32), and hydrogenase (6). A considerable level of TMAO reductase activity in strain AN180 grown without electron acceptors is due to the catalytic effect of DMSO reductase (32). Such TMAO-reducing activity was repressed in the cells grown with nitrate. The specific activity of nitrate reductase was fairly high in strain AN180 grown without nitrate. Such nitrate reductase activity is probably attributed to nitrate reductase Z which is encoded in the *narZ* gene, for whose expression nitrate is not required (4, 5); nitrate-inducible nitrate reductase is encoded in the *narGHJI* open (24).

In contrast, there were low specific activities of TMAO reductase, nitrate reductase, DMSO reductase, formate dehydrogenase N, and formate dehydrogenase H in strain TR111. When strain TR111 was grown anaerobically with TMAO, the TMAO reductase activity was less than 10% of the parent strain, though the enzyme is apparently induced. There was no induction of nitrate reductase in the nitrate culture. On the other hand, nitrate repression of fumarate reductase and hydrogenase were released in strain TR111, but there was no recovery of the activities of DMSO reductase and two formate dehydrogenases in the nitrate-grown cells. Strain HM177 also showed specific activities of enzymes similar to those in strain TR111.

These results indicate that the *torR* gene is required for activities of TMAO reductase, nitrate reductase, DMSO reductase, the two formate dehydrogenases N and H, and the nitrate repression of fumarate reductase and hydrogenase.

Ultracentrifugal precipitates from cell-free extracts were subjected to the sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Laemmli (16) and protein was stained with Coomassie blue R-250 (Fig. 1). There was no clear difference in protein band migration between the two strains grown anaerobically without electron acceptors (lanes 1 and 2). Strain AN180 contained proteins with a molecular mass of 108 kDa and 94 kDa in anaerobiosis with TMAO (lane 4) and proteins with 130 kDa and 108 kDa in nitrate culture (lane 6), but these proteins were not found in strain TR111 grown with TMAO (lane 5) and with nitrate (lane 7). These proteins of 94, 108, and 130 kDa correspond to the subunit of TMAO reductase (34), the selenopeptide of formate dehydrogenase N, and the α -subunit of nitrate reductase (9), respectively. On the other hand, a protein with 74 kDa was not produced in nitrate-grown strain AN180 (lane 6), whereas strain TR111 grown anaerobically with nitrate contained it (lane 7). This protein seems to be the subunit A of fumarate reductase (17). In addition, a new protein (20 kDa) was found in strain TR111 grown under anaerobic conditions with either TMAO or nitrate.

Since the five enzymes TMAO reductase, nitrate reductase, DMSO reductase, and the two formate dehydrogenases N and H usually contain molybdenum cofactor as a prosthetic group, the amounts of the cofactor in extracts from cells grown anaerobically with and without TMAO were determined by reconstituting *Neurospora crassa nit-1* nitrate reductase as described previously (34). One unit of

Fig. 1. SDS-polyacrylamide gel electrophoresis of the *E. coli* membrane. The gel concentration was 12.5%, and the protein was stained with Coomassie blue R-250. Cell membrane fractions were prepared from strains AN180 (lanes 1, 4, and 6) and TR111 (lanes 2, 5, and 7) grown under anaerobic conditions; without electron acceptor (lanes 1 and 2); with TMAO (lanes 4 and 5); with nitrate (lanes 6 and 7). Molecular weight markers were in lane 3. The amount of protein in each lane was about 30 μ g. The molecular weight markers are: 170 K, macroglobulin; 97.4 K, phosphorylase b; 55.4 K, glutamate dehydrogenase; 36.5 K, lactate dehydrogenase; 20.1 K, trypsin inhibitor.

molybdenum cofactor was defined as the amount that restored nitrate reductase activity to reduce 1 μ mol of nitrate per h. The contents of molybdenum cofactor were almost equal under the two growth conditions: 1.14 units/mg of protein in strain TR111 and 1.42–1.54 units/mg of protein in strain AN180. These findings indicate that molybdenum cofactor synthesis was not affected by the *torR* mutation.

In this study it was shown that *torR* mutation causes either a lack of activities of TMAO, nitrate, and DMSO reductases, and two formate dehydrogenases, or release of nitrate repression on fumarate reductase and hydrogenase. This fact indicates that the *torR* gene is involved in the regulation of the gene expression for these enzymes. The *torR* gene was located between *xyl* and *mtl*. In this region there is the *fdhA* locus which is required for the synthesis of the enzymatically active formate dehydrogenases N(10) and H(33). However, an *fdhA* mutant of *E. coli* retains nitrate reductase activity (10), unlike the *torR* mutant.

A few gene products have been known to work as transcriptional activators in expression of some gene operons for anaerobically inducible enzymes. Fnr protein is involved in expression of *narGHJI* for nitrate reductase, *frdABCD* for fumarate reductase, *nirB* for nitrite reductase and so on (8, 13, 19, 25, 26). The *ntrA* gene product (NtrA) is required for the expression of the two enzymes of the formate hydrogenlyase system formate dehydrogenase H and hydrogenase 3 (3). Involvement of the products of the *ntrBC*-like genes in the regulation of the hydrogenase 3 gene

also has been shown (29). The *narL* gene product (NarL) also acts as a positive transcriptional regulator in conjunction with Fnr on *narGHJI* (19, 21, 27) and on the *narK* gene (28) the product of which is assumed to be required for nitrate transport (22). The NarL protein is also involved in transcriptional repression of some enzyme operons in the presence of nitrate; for example, *frdABCD* (12, 14, 27) and *dmsABC* for DMSO reductase (7). The function of a product of the *torR* gene is unambiguously distinct from the Fnr, NtrA, and NarL proteins. Further investigations including analysis of molecular cloning of *torR* are expected to indicate the mechanism of transcriptional regulation for anaerobically expressible genes.

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