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# The *torYZ* (*yecK bisZ*) Operon Encodes a Third Respiratory Trimethylamine *N*-Oxide Reductase in *Escherichia coli*

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**The *bisZ* gene of *Escherichia coli* was previously described as encoding a minor biotin sulfoxide (BSO) reductase in addition to the main cytoplasmic BSO reductase, BisC. In this study, *bisZ* has been renamed *torZ* based on the findings that (i) the *torZ* gene product, TorZ, is able to reduce trimethylamine *N*-oxide (TMAO) more efficiently than BSO; (ii) although TorZ is more homologous to BisC than to the TMAO reductase TorA (63 and 42% identity, respectively), it is located mainly in the periplasm as is TorA; (iii) *torZ* belongs to the *torYZ* operon, and the first gene, *torY* (formerly *yecK*), encodes a pentahemic *c*-type cytochrome homologous to the TorC cytochrome of the TorCAD respiratory system. Furthermore, the *torYZ* operon encodes a third TMAO respiratory system, with catalytic properties that are clearly different from those of the TorCAD and the DmsABC systems. The *torYZ* and the *torCAD* operons may have diverged from a common ancestor, but, surprisingly, no *torD* homologue is found in the sequences around *torYZ*. Moreover, the *torYZ* operon is expressed at very low levels under the conditions tested, and, in contrast to *torCAD*, it is not induced by TMAO or dimethyl sulfoxide.**

*Escherichia coli* can survive in various growth conditions owing to its ability to adapt in response to environmental changes. For example, in anaerobiosis and according to the exogenous electron acceptor present in the medium, this organism synthesizes the energetically more appropriate respiratory system (18). Sometimes, more than one respiratory system is produced for a given substrate. For instance, reduction of nitrate can be carried out by at least three respiratory systems (10). At high concentrations of nitrate, only the membranous NarG system is synthesized (46), whereas at very low concentrations, the periplasmic Nap system is produced (36). The operon encoding a third system (NarZ) is expressed during the early stationary phase under control of $\sigma^s$, irrespective of the presence of nitrate (8). Accordingly, whatever the nitrate concentration and the growth phase, at least one of the nitrate reductases is synthesized in the cell (8, 36).

Trimethylamine *N*-oxide (TMAO) is reduced to the volatile compound trimethylamine (TMA) by at least two respiratory systems, the TorCAD and the DmsABC systems (5, 29). The *torCAD* operon, which encodes the periplasmic Tor system, is induced in the presence of TMAO by the TorS-TorR two-component regulatory system (25), whereas the membranous dimethyl sulfoxide (DMSO) reductase system, encoded by the *dmsABC* operon, is synthesized constitutively in anaerobiosis (5). The reason for the presence in the same host of several systems dedicated to a common substrate is still unclear, but one possibility is that they allow the cell to better adapt to changing environmental conditions during the different growth phases.

The terminal reductase of the inducible Tor pathway, TorA, encoded by the *torA* gene, is a periplasmic molybdoenzyme of 90 kDa (29) that is thought to receive electrons from the membrane pool of menaquinone through the TorC protein. TorC, which is encoded by the first gene of the *torCAD* operon, is a pentahemic *c*-type cytochrome anchored to the inner membrane by its N-terminal extremity (21, 29). The last gene of the *tor* operon, *torD*, encodes a cytoplasmic protein of 23 kDa, which is a private chaperone that is required for TorA assembly (35). TorD interacts with TorA at an early stage of TorA synthesis, probably before the insertion of the molybdenum cofactor. After folding and cofactor insertion, TorA is translocated across the inner membrane by the Tat (twin arginine translocation) system (40, 41, 52). Except for the *c*-type cytochromes (49), the metalloproteins located into the periplasm are transported by this pathway. The N-terminal signal sequences of the metalloenzymes translocated by the Tat mechanism are recognized by their length (>30 amino acids), the twin-arginine motif RRXFL, and their hydrophobicity (3, 11).

According to sequence homologies and to its biochemical properties, TorA belongs to the DMSO reductase family (20, 22). In this family of molybdoenzymes, three groups of enzymes can be defined, as follows: (i) the specific TMAO reductases encoded by *torA* of *E. coli* or *Shewanella massilia*, which do not have any *S*-oxide reductase activity and can only reduce TMAO as a natural substrate (13, 23); (ii) the TMAO-DMSO reductases like the DmsA subunit of the *E. coli* membranous DMSO reductase or the periplasmic DorA and DmsA enzymes of *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, respectively, which are able to reduce a wide range of *N*- and *S*-oxide compounds, including DMSO and TMAO (42, 45); and (iii) the biotin *d*-sulfoxide reductases like BisC from *E. coli* and the biotin sulfoxide (BSO) reductase from *R. sphaeroides*, which are cytoplasmic enzymes involved primarily in the recycling of biotin from BSO (33, 34). An in vitro study showed that the *R. sphaeroides* enzyme can also poorly reduce other *N*- and *S*-oxides like TMAO and DMSO (17, 34). Although these three groups of enzymes share sequence homologies, a major difference is that enzymes of the first two groups are involved in anaerobic respiratory processes while the cytoplasmic BSO reductase enzymes are not.

Recently, it has been proposed that a newly characterized gene, *bisZ*, encodes a second BSO reductase in *E. coli* (12). Genetic and biochemical evidence showed that the *bisZ* product was responsible for the 4% background BSO reductase

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TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Genotype(s) and/or characteristic(s) | Reference or source |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| MC4100 | *araD139* Δ(*lacIPOZYA-argF*)*UI69 rpsL thi* | M. J. Casadaban |
| LCB620 | MC4100 *tor*A8::MudII 1734 (*torA′-′lacZ*) | 29 |
| LCB502 | MC4100 *tor*C2::ΩSpc$^r$ | 1 |
| DSS401 | MC4100 Δ*dms* Km$^r$ | 38 |
| LCB504 | LCB502 Δ*dms* Km$^r$ Spc$^r$ | This work |
| B1LK0 | MC4100 Δ*tatC* | 7 |
| Plasmids | | |
| pJF119EH | Vector containing the $P_{tac}$ promoter | 16 |
| pBAD24 | Vector containing the $P_{bad}$ promoter | 19 |
| pGE593 | Operon fusion vector | 14 |
| pEC86 | *ccmABCDEFGH* inserted into pACYC184 | 2 |
| ptorCAD | *torCAD* coding sequence inserted into pJF119EH | This work |
| ptorZ | *torZ* coding sequence inserted into pJF119EH | This work |
| ptorYZ | *torYZ* (*yecK bisZ*) coding sequence inserted into pJF119EH | This work |
| ptorY | *torZ* gene partly deleted from ptorYZ | This work |
| pBtorYZ | *torYZ* (*yecK bisZ*) coding sequence inserted into pBAD24 | This work |
| pPTorYZ | *torY* promoter region (−283 to +170)[a] cloned into pGE593 | This work |

[a] Nucleotide positions relative to the first nucleotide of the initiation codon of *torY*.

activity observed in a *bisC* mutant. Moreover, the *bisZ* gene encodes a protein that exhibits 62% sequence identity with the *bisC* product. We demonstrate in this paper that, in contrast to BisC, the *bisZ* gene product is periplasmically located. Furthermore, this enzyme has an extended substrate specificity which includes, in addition to BSO, other *N*- and *S*-oxides, such as TMAO. Significantly, it exhibits greater catalytic activity with TMAO than with BSO. Accordingly, we propose to rename *bisZ* as *torZ*. We also show that *torZ* (*bisZ*) is the second gene of the *torYZ* (*yecK bisZ*) operon and that *torY* encodes a *c*-type cytochrome homologous to TorC. Finally, TorY and TorZ constitute a respiratory system.

## MATERIALS AND METHODS

**Chemicals.** The *N*- and *S*-oxide compounds were purchased from Sigma or Aldrich except for *d*-biotin *d*-sulfoxide, which was synthesized from biotin as described by Melville (28).

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this work are listed in Table 1. To maintain selection for plasmids or to select for transductant strains, we used antibiotics as follows: ampicillin, 50 μg/ml; chloramphenicol, 10 μg/ml; kanamycin, 50 μg/ml; and spectinomycin, 25 μg/ml. For the biochemical study, cells were grown anaerobically at 37°C on Luria-Bertani (LB) media. The concentration of arabinose or of glucose, added in the growth medium, is detailed for each experiment in Results. Otherwise, growth of *E. coli* was performed under anaerobic conditions in 3-ml full-cap cuvettes at 37°C with a minimal salt medium (MSM) derived from that described by Bilous and Weiner (4). It contained $K_2HPO_4$ (3.5%); $KH_2PO_4$ (1%), $(NH_4)_2SO_4$ (0.5%), $MgSO_4$ (0.05%), $CaCl_2$ (0.015%), Na citrate (0.3%), casein acid hydrolysate (0.15%; Difco), and thiamine hydrochloride (0.002%, pH 7). The MSM was supplemented with 0.5% glycerol as a nonfermentable carbon source and with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when necessary. Electron acceptors were used at a concentration of 50 mM. The MSM was inoculated at a dilution of 1% with cells grown overnight in LB broth (supplemented with ampicillin when necessary), centrifuged, and resuspended in the same volume of MSM. Growth was monitored in the same full-capped cuvettes at 600 nm. Values (about 0.15 after 24 h) obtained from a control curve with cells in MSM supplemented with glycerol but without electron acceptor were subtracted from the experimental values.

**DNA manipulations.** DNA was prepared with the High Pure DNA Isolation kit from Boehringer Mannheim. Plasmid preparation, restriction endonuclease digestions, and DNA purification and ligation were carried out as described by Sambrook et al. (39). Transformations were performed according to the method of Chung and Miller (9). PCR amplification was carried out using standard procedures according to the supplier's instructions. Reverse transcriptase-PCR (RT-PCR) was performed with the Promega Access system. The oligonucleotides used were as follows: a, 5′-ATTAGAGGAATCTATGCGAGGGAAAAAACG-3′; b, 5′-GTGGACAGTTCCCTGATATTCCTC- 3′; c, 5′-CGCTATGGCAATACGCTGAAAAACTTG-3′; d, 5′-TAACAATTGACCATGATCAGGGAGGAAGTTATGACATTAAC-3′; and e, 5′-CCGCCGCCGTAGACTGTAAGGAAT-3′. One microgram of total RNA prepared with the High Pure RNA Isolation kit (Boehringer Mannheim) was denatured at 94°C for 2 min in the presence of either primers a and b, primers c and e, or primers d and e. Immediately afterward, reverse transcription and 35 cycles of PCR amplification were carried out according to the supplier's protocol.

**Construction of plasmids.** To create plasmids ptorZ and ptorYZ, we used PCR to generate DNA fragments corresponding to the *torZ* and the *torYZ* coding sequences with, in both cases, an upstream *Mfe*I site and a downstream *Sma*I site. After enzymatic hydrolysis, the PCR products were cloned into the compatible *Eco*RI and *Sma*I sites of pJF119EH (16), yielding plasmids ptorZ and ptorYZ. In these plasmids, *torZ* and *torYZ* are under the control of the $P_{tac}$ promoter. To create the pBtorYZ, the same *torYZ* PCR product was cloned into the *Eco*RI and *Sma*I sites of pBAD24 (19). In this construct, the *torYZ* genes are under the control of the arabinose promoter. To create plasmid ptorY, the *torZ* gene was partly deleted from plasmid ptorYZ after an *Eco*RI digestion, followed by an intramolecular ligation. To create plasmid ptorCAD, we used PCR to generate a promoterless *torCAD* DNA fragment. This purified PCR product was then cloned into the pPCR-script vector (Stratagene), according to the supplier's protocol, to yield pPStorCAD. The *Pst*I-*Sac*I fragment from pPStorCAD was cloned into the same sites on pJF119EH, resulting in plasmid ptorCAD, in which the entire *tor* operon is under the control of the $P_{tac}$ promoter. To create plasmid pPTorYZ, we amplified the *torY* promoter region by PCR (from position −283 to position +170 relative to the first nucleotide of the initiation codon of *torY* gene). The DNA fragment was blunted using the blunting kit from Takara and introduced into plasmid pGE593 (14), previously linearized by *Sma*I, thus placing the *lacZ* gene under the control of the putative *torY* promoter. All the PCR products and fusion sites were confirmed by sequencing, except for the *torCAD* coding region of ptorCAD which was subjected to a PCR amplification of only 13 cycles in order to minimize the number of possible mutations.

**Construction of strain LCB504.** The mutation of strain DSS401 (Δ*dms* Km$^r$) was transferred to LCB502 by P1 transduction, resulting in a *torC dms* strain (LCB504). Integration of the Km$^r$ gene at the correct position on the chromosome was verified by PCR.

**β-galactosidase assays.** Strain LCB504 carrying pPTorYZ was grown anaerobically at 37°C in LB medium alone or supplemented with 50 mM TMAO, DMSO, or BSO. β-galactosidase activities were measured according to the method of Miller (30) from culture samples that were taken during the exponential- and the stationary-growth phases.

**Preparation of subcellular fractions.** Crude extracts were prepared by disrupting the cells in a French press as described by Iobbi-Nivol et al (24). The periplasmic fractions were prepared according to the sucrose-lysosyme-EDTA procedure described by Osborn et al. (32). Membranous and cytoplasmic fractions were obtained from the spheroplasts after disruption in a French press and ultracentrifugation as detailed by Silvestro et al. (44).

**Enzyme purification.** TorZ (BisZ) was purified from the periplasm fraction obtained from 4 g of LCB620/pBtorYZ cells grown anaerobically in the presence of 0.1% arabinose and ampicillin. The periplasm was dialyzed to remove the sucrose and applied to an ion-exchange Q Sepharose, HiLoad 16/10 column (Pharmacia-Biotech). The fractions obtained from a 0 to 0.5 M NaCl linear gradient elution were assayed for TMAO reductase activity, and active fractions were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (26). Two fractions, possessing also the highest specific activity, presented only two Coomassie blue-stained bands each. The major one, corresponding to more than 80% of the stained proteins, had a calculated molecular mass of about 90 kDa, whereas the minor band was slightly smaller. These fractions were then tested for TMAO and DMSO reductase activities on SDS gels according to the procedure described by Pommier et al. (35). The single active band obtained for both the TMAO and DMSO activities corresponded to the major Coomassie blue band. The two active fractions were pooled and used for analytical experiments. A total quantity of 1.76 mg of protein was obtained with a specific activity of 709 μmol of TMAO reduced per min per mg of protein.

**Analytical procedures.** TMAO reductase activity was measured spectrophotometrically at 37°C by following the oxydation of reduced benzyl viologen at 600 nm coupled to the reduction of TMAO. Each of the compounds tested (see Table 4 for a list) was prepared at a final concentration of 1 or 2 M and used at least at eight different concentrations with three to four duplicates. Kinetic parameters were determined as previously described (23).

The N-terminal sequence of TorZ (BisZ) was determined by Edman degradation (model 470a; Applied Biosystems) after electroblotting of TorZ onto polyvinylidene difluoride membrane (50).

The plasmid pEC86, carrying all the *ccm* genes, whose products are involved

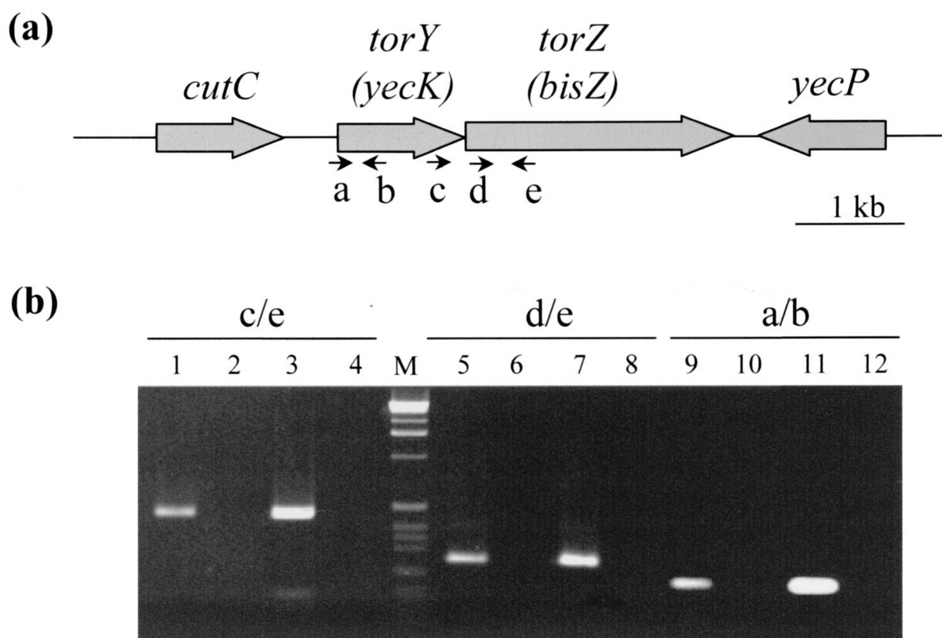


FIG. 1. (a) Physical map of the *torYZ* operon region of *E. coli.* The large arrows show the locations and the orientations of the reading frames. The primers (a, b, c, d, and e) used for the RT-PCR are indicated. (b) Analysis of *tor* gene transcription by RT-PCR was followed by 2% agarose gel electrophoresis. The RT-PCR was carried out with primers c and e (lanes 1 to 4), primers d and e (lanes 5 to 8), or primers a and b (lanes 9 to 12). Lanes 1, 5, and 9, RT-PCR experiments with 1 μg of *E. coli* total RNA; lanes 2, 6, and 10, same experiments as described for lanes 1, 5, and 9 but without reverse transcriptase to check the absence of DNA traces in the RNA preparation; lanes 3, 7, and 11, control PCR with genomic DNA; lanes 4, 8, and 12, the same experiments as described for lanes 1, 5, and 9 but without RNA (negative control); lane M, DNA ladder corresponding to the 1-kb ladder of Gibco BRL.

in the maturation mechanism of the *c*-type cytochromes, was introduced into the LCB620/pBtorYZ strain to increase the amount of mature TorY (2). The presence of covalently bound hemes in *c*-type cytochromes was revealed by staining for peroxidase activity using 3,3′,5,5′-tetramethylbenzidine (TMBZ) (48). Protein concentration was estimated by the procedure of Lowry et al. (27).

## RESULTS

**Gene organization and putative products of the *bisZ* locus.** The *torZ* (*bisZ*) gene is located at kb 1955 on the chromosome of *E. coli.* The analysis of the DNA sequences surrounding this gene revealed the presence of a 1-kb open reading frame that we propose to call *torY* (formerly designated *yecK*), located immediately upstream from *torZ* (*bisZ*) and transcribed in the same orientation (Fig. 1). The distance between *torY* (*yecK*) and *torZ* (*bisZ*) is only 24 bp, while a noncoding region of 387 bp separates *torY* (*yecK*) from the upstream *cutC* gene which is transcribed in the same orientation and ends with a probable transcriptional terminator. The next gene downstream from *torZ* (*bisZ*) (*yecP*) is transcribed divergently. To determine whether the *torY* (*yecK*) and *torZ* (*bisZ*) genes are organized in a single transcription unit, RT-PCR was performed using RNA extracted from a strain grown anaerobically and appropriate oligonucleotide pairs that hybridize to regions in the beginning or within *torY* (*yecK*) and within *torZ* (*bisZ*), as shown in Fig. 1. The PCR synthesis of a DNA fragment that overlaps the region between the end of *torY* (*yecK*) and the beginning of *torZ* (*bisZ*) strongly suggests that these genes are organized in an operon. Amplification of DNA fragments that cover the beginning of *torY* (*yecK*) indicates that the transcriptional start of this operon is, as expected, upstream from the ATG start codon of *torY* (*yecK*). The intergenic region located between the *cutC* and the *torY* (*yecK*) genes was cloned upstream from the promoterless *lacZ* gene of the multicopy plasmid pGE593 to yield plasmid pPTorYZ. Unfortunately, the β-galactosidase activity measured from strain MC4100 carrying pPTorYZ was very low (10 to 20 Miller units) under all the growth conditions tested (see Materials and Methods). This result suggests either that the *torYZ* (*yecK bisZ*) operon is always expressed at a very low level or that the conditions for inducing this operon have not been discovered.

Nucleotide analysis indicates that a strong ribosome binding site (GAGGA) is located 9 bp upstream from the start codon of both *torY* (*yecK*) and *torZ* (*bisZ*). The sequence of the *torY* (*yecK*) gene product (TorY) revealed five consensus heme binding sites (CXXCH); thus, TorY is likely to be a pentahemic *c*-type cytochrome. The presence of a hydrophobic segment of 20 residues located in the N-terminal extremity of TorY suggests that TorY is anchored to the inner membrane. TorY is homologous to TorC and DorC (36 and 37% identity, respectively), and, like TorC and DorC, it seems to contain two domains: an N-terminal tetrahemic domain presenting homologies with the members of the NirT family and a C-terminal monohemic domain which appears only in *c*-type cytochromes involved in TMAO and DMSO respiratory systems (13, 31, 37).

As previously described by del Campillo-Campbell and Campbell (12), the primary amino acid sequence of the *torZ* (*bisZ*) gene product shares homologies with the DMSO reductase family of the molybdoenzymes (20). The best score in the homology search was observed with the cytoplasmic BSO reductase of *E. coli* (62% identity) (33). Moreover, the *torZ* (*bisZ*) product exhibited high similarity scores with periplasmic molybdoenzymes like DorA from *R. capsulatus*, DmsA from *R. sphaeroides*, TorA from *E. coli*, and TorA from *S. massilia* (46, 46, 42, and 38% identity, respectively) (13, 29, 43, 53). Interestingly, a significant difference between the sequences of the *torZ* (*BisZ*) product and BisC, which was not previously pointed out, is that the N-terminal part of TorZ (BisZ) shows the characteristics of a signal peptide specifically found in molybdoenzymes such as TorA, DorA, and DmsA, (Fig. 2) (3). The presence of a motif RRXFI, close to the classical twin-

RsDMSA MTKLSGQELHAELS**RRAFL**SYTAAVGALGLCGTSLLAQG*ARA* 42
RcDorA MTKFSGNELRAELY**RRAFL**SYSVAPGALMFGRSLLAKG*ARA* 41
SmTorA MN**RRDFL**KGIASSSFVVLGGSSVLTPLN*ALA* 31
EcTorA MNNNDLFQAS**RRRFL**AQLGGLTVAGMLGPSLLTPRR*ATA* 39
HibisZ MKKNNVNEQ**RRDFL**KKTSLGVAGSALSGGMVGVVSKS*AVA* 40
EcTorZ MTLT**RREFI**KHSGIAAGALVVTSAAPL*AWA* 30

FIG. 2. Amino acid sequence alignment of the N-terminal part of the DMSO-TMAO reductases of *R. sphaeroides* (RsDmsA) (53) and *R. capsulatus* (RcDorA) (43), the TMAO reductases of *S. massilia* (SmTorA) (13) and *E. coli* (EcTorA) (29), the product of the *H. influenzae bisZ* gene (HibisZ) (47), and the product of the *E. coli torZ* (*bisZ*) gene (EctorZ). The signal sequence cleavage site and the redox protein export conserved motif are in italic and in bold type, respectively.

arginine motif (RRXFL), followed by a long hydrophobic segment and by the consensus AXA cleavage site led us to propose that, in contrast to the cytoplasmic BisC protein, the product of *torZ* (*bisZ*) is exported to the periplasm.

This analysis allowed us to conclude that the two genes, *torY* (*yecK*) and *torZ* (*bisZ*), which appear to be organized in a single transcription unit, should encode a membrane-bound pentahemic cytochrome and an unexpected periplasmic molybdoprotein, respectively. The homologies observed between the components of the electron transfer chain of both the Tor and Dor respiratory systems and the products of this new operon suggested to us that it may encode a novel respiratory system reducing *N*- and/or *S*-oxide compounds.

***torYZ* (*yecK bisZ*) operon encodes a respiratory system.** To test our hypothesis that the TorY and TorZ proteins are involved in an electron transfer pathway similar to that of the TorCAD system, a strain that was unable to reduce TMAO was constructed. This strain, LCB504, carries an interposon in the beginning of the first gene of the *torCAD* operon and a deletion of the entire *dmsABC* operon (Table 1). As expected, strain LCB504, containing the pJF119 expression vector, grew at an extremely slow rate under anaerobic conditions with TMAO as the only exogenous electron acceptor (Fig. 3). When

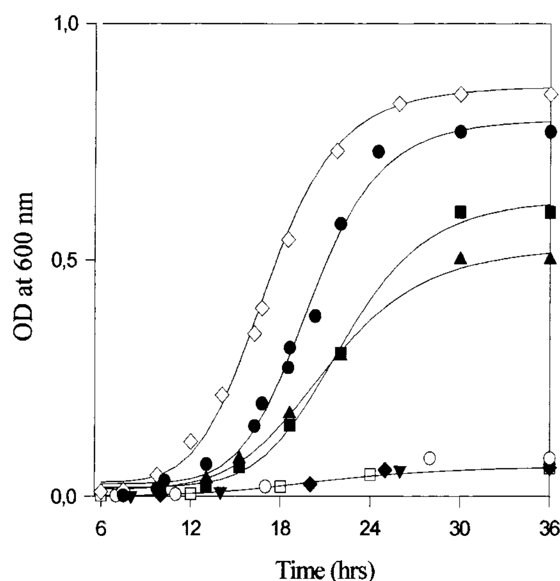


FIG. 3. Anaerobic growth profiles of *E. coli* strain LCB504 carrying plasmid ptorYZ or plasmid ptorCAD. The LCB504 recombinant strains were grown in MSM in the presence of 1 mM IPTG (unless otherwise indicated) and of a 50 mM concentration of either TMAO (strains carrying ptorCAD [◇], ptorYZ [●], ptorYZ but without IPTG [○], pJF119EH [□], ptorY [◆], or ptorZ [▼]), DMSO (strain carrying ptorYZ [▲]), or BSO (strain carrying ptorYZ [■]). Growth was monitored at 600 nm as described in Materials and Methods. Data are typical of those obtained from at least three independent experiments. OD, optical density.

TABLE 2. Anaerobic growth of LCB504 carrying either ptorYZ or ptorCAD with *N*- and *S*-oxide compounds

| Compound | Result for strain[a]: | |
|---|---|---|
| | LCB504/ptorYZ | LCB504/ptorCAD |
| None | −[b] | − |
| *N*-oxide compounds | | |
| TMAO | ++ | ++ |
| 2-Picoline *N*-oxide | ± | − |
| 4-Methylmorpholine *N*-oxide | + | ++ |
| *S*-oxide compounds | | |
| DMSO | + | − |
| BSO | + | − |
| DL-Methionine sulfoxide | + | − |
| TMSO | ± | − |

[a] The growth conditions are identical to those described for Fig. 3.
[b] ++, growth profile similar to curves obtained with TMAO (Fig. 3); +, growth profile similar to curves obtained with DMSO or BSO (Fig. 3); −, growth profile similar to curve obtained without IPTG (Fig. 3); ±, growth significant but low compared to that obtained with DMSO or BSO (Fig. 3).

plasmid ptorCAD, carrying the *torCAD* operon under the control of the $P_{tac}$ promoter, was introduced into strain LCB504, the recombinant strain exhibited a high growth rate in the presence of IPTG. Introduction of plasmid ptorYZ, carrying the *torYZ* (*yecK bisZ*) operon under the control of the $P_{tac}$ promoter, into strain LCB504 also yielded a recombinant strain with similar IPTG-dependent growth rate (Fig. 3). In the absence of IPTG, no significant growth was observed with either recombinant strain (Fig. 3). It is noteworthy that production of the characteristic odor of volatile TMA occurred under condition of rapid growth, indicating that TMAO was reduced to TMA in both experiments. Therefore, when expressed to a certain level, the *torYZ* (*yecK bisZ*) operon, like *torCAD,* allows the strain to use TMAO as a substrate for anaerobic respiration.

Because the *torZ* (*bisZ*) gene product was previously shown to encode a BSO reductase enzyme (12), we also tested the growth of strain LCB504 carrying ptorYZ when BSO was added to the medium. Again, growth was observed, but it did not reach either the rate or the yield obtained with TMAO, indicating that TMAO serves as a better electron acceptor than BSO for the terminal reductase under these conditions (Fig. 3). This result confirms that BSO can be reduced by the TorZ (BisZ) enzyme. In contrast, the expression of the plasmid born *torCAD* operon allowed no bacterial growth in the presence of BSO (Table 2), suggesting that the specificity of TorA and TorZ are quite different.

As shown previously, unlike the membranous DMSO reductase and the *Rhodobacter* DorA enzyme, TorA is unable to reduce DMSO (23, 42, 43, 45). As expected, when the strain LCB504 harboring plasmid ptorCAD was grown in minimal media in the presence of DMSO, no growth was detected (Table 2). In contrast, when the strain carried the ptorYZ plasmid, the bacteria grew quite well (Fig. 3), confirming that the TorYZ system has a substrate specificity broader than that of TorCAD. To extend this analysis, we compared the effect of various *N*- and *S*-oxide substrates on the bacterial growth of strain LCB504 carrying either ptorYZ or ptorCAD (Table 2). Expression of *torYZ* allows *E. coli* to grow anaerobically on a wider range of substrates than does expression of *torCAD.*

To establish that the TorY cytochrome is required for electron transfer to the TorZ (BisZ) terminal enzyme, we cloned the *torZ* (*bisZ*) gene alone under the control of the $P_{tac}$ promoter. The strain carrying the ptorZ plasmid did not grow in

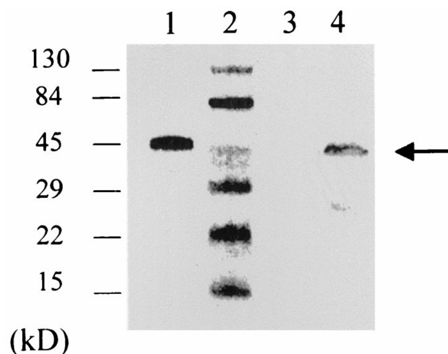


FIG. 4. Detection of *c*-type cytochromes by TMBZ staining. Membranous proteins (50 μg) from cells carrying pBtorYZ grown anaerobically at 37°C in LB medium supplemented with 0.2% glucose (lane 3) or supplemented with 0.2% arabinose (lane 4) were loaded on an SDS-10% polyacrylamide gel. As a control, purified TorC (2.5 μg) was also loaded onto the gel (lane 1). Sigma Wide Range color markers were also used (lane 2). Samples were heated prior to loading, and after electrophoresis, the gel was stained for heme with TMBZ. The arrow indicates the position of the *torY* gene product.

the presence of TMAO, DMSO, or BSO (Fig. 3 and data not shown). The *torY* (*yecK*) product is thus required in the respiration pathway. Similarly, the *torZ* (*bisZ*) gene of ptorYZ was inactivated, leading to plasmid ptorY, and, as expected, no significant growth was allowed (Fig. 3).

This set of in vivo experiments clearly shows that, in contrast to the BisC enzyme, the *torZ* (*bisZ*) product together with the *torY* (*yecK*) product is involved in an anaerobic respiratory system. Moreover, this system can use several exogenous electron acceptors, including TMAO, BSO, and DMSO. As TMAO seems to be the most efficient substrate for this respiratory system, we conclude its operon should be called the *torYZ* operon.

**TorYZ respiratory system is made up of a membrane-anchored cytochrome and a periplasmic reductase.** For the production of both the *torY* (*yecK*) and *torZ* (*bisZ*) products, the genes were cloned under the strict control of the $P_{bad}$ promoter into the pBAD24 vector. The resulting plasmid, pBtorYZ, was then introduced into strain LCB620 and grown in the presence of either arabinose or glucose for the induction or repression, respectively, of *torYZ* expression. First, to confirm the predictions deduced from the sequence analysis about the cellular location of TorY as well as the presence of heme in it, various fractions of the strain, carrying both plasmids pBtorYZ and pEC86, grown in presence of 0.2% arabinose or 0.2% glucose were tested by SDS-PAGE followed by heme staining. In the membrane fraction of cells grown in the presence of arabinose, a cytochrome of about 40 kDa was observed (Fig. 4). It is distinguishable from TorC by its slightly smaller size, and it is absent when glucose is added to the growth media or in the soluble extracts of the bacteria (Fig. 4 and data not shown). Only five *c*-type cytochromes have been described previously for *E. coli*: two of them are involved in the periplasmic nitrate respiratory system (Nap), two others are involved in the nitrite reduction pathway (Nrf), and one, TorC, belongs to the TMAO respiration system (21). This study shows that the *E. coli* genome contains a gene, *torY,* able to encode a sixth *c*-type cytochrome. According to the results obtained from a search in the Colibri databank using the heme binding motif as a pattern, no other multihemic cytochrome seems to be encoded by the *E. coli* genome.

Results obtained during the growth experiments led us to test the *torZ* (*bisZ*) gene product for in vitro TMAO reductase activity. As expected, a significant TMAO reductase activity was measured in the supernatant of cells carrying pBtorYZ grown in the presence of 0.1% arabinose (5.5 μmol/min/mg of protein), whereas when 0.1% glucose was added to the growth media or when the strain contained only the vector, no significant activities were observed (0.05 and 0.02 μmol/min/mg of protein, respectively). These results support the idea that TorZ (BisZ) is a soluble enzyme.

To distinguish the location of this protein in the cell, periplasmic and cytoplasmic fractions were prepared from cells grown in the presence of arabinose. A total of 80% of the TMAO reductase activity was recovered in the periplasmic fraction of the cell (Table 3). This result is in agreement with the presence of a putative signal sequence in the N-terminal part of TorZ (BisZ). To determine the position of the cleavage site, the N-terminal extremity of TorZ (BisZ) was sequenced after purification of the protein from the periplasmic fraction. The obtained sequence (EEKGGKIL) corresponds to positions 31 to 39 of the deduced amino acid sequence and follows the consensus AXA cleavage site, as indicated in Fig. 2. Usually, the periplasmic metalloenzymes possessing the twin-arginine motif are transported across the inner membrane by the Tat system (41). To show that translocation of TorZ (BisZ) involves the Tat pathway, a *tatC* strain (B1LK0) lacking the pore-forming protein, was transformed by plasmid pBtorYZ. We observed that, in this recombinant strain grown with arabinose, the TMAO reductase activity in the periplasm is about 16 times lower than that in the wild type (Table 3). Moreover, most of the activity is found in the cytoplasm in the *tat* strain. Therefore, the transport of this enzyme across the membrane is dependent on the Tat system.

Taking these results altogether, it appears that, in contrast to the BisC protein, the *torZ* (*bisZ*) product is located in the periplasm and involved with TorY in a respiratory process. All these findings strengthen the idea that this new respiratory system resembles the TorCAD system. However, the fact that the TorYZ system allows growth in the presence of *S*-oxides suggests that the catalytic properties of TorZ (BisZ) are quite different from those of TorA (23).

**Catalytic properties of TorZ (BisZ).** The kinetic study was performed on the purified product of *torZ* (*bisZ*) as described in the Materials and Methods. The compounds tested were structurally related to TMAO or DMSO and have been previously used to determine the specificity of TorA and the membranous DMSO reductase of *E. coli* (23, 45).

As shown in Table 4, the best catalytic efficiency ($V_{max}/K_m$),

TABLE 3. Repartition of TMAO reductase activity in strains carrying the pBtorYZ plasmid

| Strain | Inducing conditions[a] | Fraction[b] | TMAO reductase activity[c] |
|---|---|---|---|
| LCB620/pBtorYZ | + | Cytoplasm | 26 |
| | + | Periplasm | 101 |
| | − | Cytoplasm | 0.7 |
| | − | Periplasm | 4.1 |
| B1LK0/pBtorYZ | + | Cytoplasm | 78 |
| | + | Periplasm | 6.4 |

[a] Cells were grown as indicated in Materials and Methods, in the presence of either 0.1% arabinose (+) for induction of the *torYZ* genes carried by the plasmid or 0.1% glucose (−) for repression.

[b] For all the strains, the periplasmic and cytoplasmic fractions were prepared under exactly the same conditions from 0.5 g of cells.

[c] The TMAO reductase activity was measured in vitro using benzyl viologen as the electron donor. Values are in micromoles of TMAO reduced per minute per gram of cells.

TABLE 4. Kinetic parameters with different electron acceptors catalyzed by the *torZ* (*bisZ*) product

| Compound[a] | $V_{max}$ (M · s$^{-1}$) | $K_m$ (mM) | $V_{max}/K_m$ |
|---|---|---|---|
| TMAO | 166 | 1.44 | 115 |
| 4-Methylmorpholine-*N*-oxide | 121 | 1.4 | 86 |
| 2-Picoline-*N*-oxide | 13.8 | ND[b] | ND |
| Pyridine-*N*-oxide | 4.21 | ND | ND |
| DMSO | 9 | ND | ND |
| TMSO | 18 | 3.37 | 5 |
| BSO | 18 | 0.36 | 50 |
| DL-Methionine sulfoxide | 11.6 | 0.8 | 14 |
| $NO_3$ | 0.46 | ND | ND |
| $NO_2$ | 0.25 | ND | ND |

[a] Assays were performed in 100 mM phosphate buffer (pH 6.8), with the same enzyme concentration.

[b] ND, values obtained were too low for a $K_m$ calculation.

which takes into account both the affinity of the enzyme for the substrate and the substrate turnover, is obtained for TMAO, 4-methylmorpholine *N*-oxide, and BSO, in decreasing order. The details of the analysis indicate that although the $K_m$ obtained with TMAO is higher than that obtained with BSO, the catalytic efficiency measured with TMAO is more than 2 times higher than that measured with BSO. This is due to the fact that the $V_{max}$ measured with TMAO is about 10 times higher than that observed with BSO. We can then conclude that TMAO is a more efficient substrate than BSO. This conclusion accommodates the differences in growth rate observed in the presence of TMAO, BSO, and 4-methylmorpholine *N*-oxide (Fig. 3 and Table 2).

Except for TMAO and 4-methylmorpholine *N*-oxide, which both appear to be good substrates for TorZ (BisZ), the *N*-oxide compounds tested in this experiment are not very efficiently reduced by TorZ (BisZ). Nitrate and nitrite, which are widespread *N*-oxide compounds, have also been tested, but neither was reduced at a significant rate by TorZ (BisZ) (Table 4). These results are reminiscent of those obtained during the kinetic study of TorA (23). Nevertheless, in contrast to TorA, the *torZ* (*bisZ*) product is capable of sulfoxide reduction. Indeed, the kinetic parameters determined for BSO, tetramethylene sulfoxide (TMSO), and DL-methionine sulfoxide indicate that TorZ (BisZ) can reduce these compounds, and among them, BSO is the best substrate. However, the TMAO reduction catalytic efficiency is never reached with any *S*-oxide. DMSO was so weakly reduced that kinetic parameters for the enzyme could not be determined without ambiguity. This was surprising, since a significant growth rate was observed with this compound (Fig. 3 and Table 2). To investigate this apparent discrepancy, a competition experiment was carried out to determine whether DMSO can bind the active site of the enzyme. The thermodynamic parameters of the TMAO reductase activity of TorZ (BisZ) were modified when DMSO (11.4 mM) was added in the assay (${K_m}_{TMAO}^{DMSO}$ = 41 mM, ${V_{max}}_{TMAO}^{DMSO}$ = 120 M s$^{-1}$). Therefore, DMSO, which is a weak substrate for the enzyme in vitro, acted as a competitive inhibitor towards TorZ. These results fit a mixed-alternative-substrate model (data not shown) (15) and emphasized the difference in substrate specificity between this enzyme and TorA since DMSO does not compete with TMAO in the catalytic site of TorA (23).

## DISCUSSION

During this study, we have demonstrated that the *torYZ* (*yecK bisZ*) operon of *E. coli* encodes a new respiratory system. This system is made up of a membranous pentahemic *c*-type cytochrome and a periplasmic molybdoreductase, and it is closely related to the Tor and Dor respiratory systems of *E. coli* and *Rhodobacter* species (29, 31, 51). The association of a pentahemic membrane-bound cytochrome with a periplasmic enzyme seems to be the hallmark of respiratory systems capable of reducing either specifically TMAO (the Tor system of *E. coli* and of *S. massilia*) or TMAO and related *N*- and *S*-oxide compounds (the Dor system of *Rhodobacter* species). Accordingly, the best substrate among those tested for the TorYZ is TMAO. However, our in vitro and in vivo studies revealed that the specificity of TorZ is different from that of either TorA or DorA. In contrast to TorA, TorZ can reduce other *N*- and *S*-oxide compounds such as BSO, and in contrast to DorA, the best substrate for TorZ is not a sulfoxide compound but TMAO.

To avoid any confusion, we proposed to rename BisZ as TorZ because this enzyme is a better TMAO reductase than a BSO reductase and because it is located in the periplasm, whereas BisC (the higher TorZ [BisZ] homologue) is a cytoplasmic protein which is not involved in a respiratory process. Our data are in agreement with those of del Campillo-Campbell and Campbell (12), showing that TorZ can reduce BSO quite efficiently, but, from our results, it is probable that reduction of BSO takes place mainly in the periplasm rather than in the cytoplasm. It would be interesting to test whether TorZ can also reduce BSO when located in the cytoplasm. If this happens, the electron donor could be different from TorY which, most probably, faces the periplasm.

In *E. coli*, two homologous nitrate respiratory systems, nitrate reductase G and nitrate reductase Z, have been described as having issued from a duplication of ancestral genes (6, 24). For a long time, the *narZYWV* operon encoding nitrate reductase Z was supposed to be expressed constitutively at a very low level. However, a recent study showed that expression of this operon is induced in the early stage of the stationary phase of cell growth in a RpoS-dependent manner (8), while the nitrate reductase G is synthesized in anaerobiosis when a high concentration of nitrate is available (10, 36, 46). The *torYZ* operon originates probably from a duplication of the *torCAD* genes. In the case of the TorYZ system, no obvious regulation has been highlighted so far. In particular, the expression of the *torYZ* operon was very low whatever the growth phase and did not increase in the presence of TMAO, DMSO, or BSO. One can imagine that either the *torYZ* operon is constitutively expressed at a very low level or the induction conditions of its expression are still unknown. This raises the question of the existence of an unknown inducer for the *torYZ* operon which might be the best natural substrate of this respiratory system. The fact that the specificity of the TorYZ system is not exactly that of the TorCAD or the DmsABC system of *E. coli* suggests that the TorYZ system has evolved from the TorCAD system to play a specific role in *E. coli* respiration. It would be interesting to see whether related bacteria, such as *Salmonella* species, contain a TorYZ homologue and, if present, how this system is expressed. In this line of thought, a gene homologous to *torZ* (*bisZ*) was found in the chromosome of *Haemophilus influenzae* (47). This putative gene has been called *bisZ*, but the presence of a potential signal sequence in the N-terminal extremity of its deduced amino acid sequence indicates that it encodes a periplasmic protein (Fig. 2). Moreover, the presence before it of a gene encoding a pentahemic cytochrome homol-

ogous to TorC and to TorY raises the question of a possible respiratory role of this *bisZ* product.

An obvious difference between the *torYZ* and the *torCAD* operons is that a *torD* gene homologue is not found in the former. TorD is a private chaperone for the terminal reductase TorA, and its absence in a *torD* strain led to a significant decrease in the quantity of the TorA protein compared to that observed with a wild-type strain, but even in such a mutant strain, 30% of the active TorA protein is present in the periplasm (35). Our result indicates that TorZ is synthesized and folded in a *torCAD* strain. Thus, TorD is not required for TorZ maturation. Therefore, TorZ folding involves either no chaperone or a TorD homologue whose gene is located elsewhere on the chromosome. Interestingly, we have found several genes which could encode TorD homologues on the *E. coli* chromosome (unpublished results). We are investigating whether one of these TorD homologues plays the role of a TorZ chaperone.

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