TorD, A Cytoplasmic Chaperone That Interacts with the Unfolded Trimethylamine N-Oxide Reductase Enzyme (TorA) in *Escherichia coli*†‡

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In *Escherichia coli*, the main anaerobic respiratory pathway responsible for reduction of trimethylamine N-oxide (TMAO) to TMA (trimethylamine) involves the terminal molybdoenzyme TorA, located in the periplasm, and the membrane anchored c-type cytochrome TorC. In this study, the role of the TorD protein, encoded by the third gene of the *torCAD* operon, is investigated. Construction of a mutant, in which the *torD* gene is interrupted, showed that the absence of TorD protein leads to a two times decrease of the final amount of TorA enzyme. However, specific activity and biochemical properties of TorA enzyme were similar to those of the enzyme produced in the wild type. Excess of TorD protein restores the normal level of TorA enzyme, and also, leads to the appearance of a new cytoplasmic form of TorA on SDS-polyacrylamide gel electrophoresis under gentle conditions. This probably indicates a new folding state of the cytoplasmic TorA protein when TorD is overexpressed. BIACore techniques demonstrated direct specific interaction between the TorA and TorD proteins. This interaction was enhanced when TorA was previously unfolded by heating. Finally, as TorA is a molybdoenzyme, we demonstrated that TorD can interact with TorA before the molybdenum cofactor has been inserted. As TorD homologue encoding genes are found in various TMAO reductase species,2 it was proposed to be a chaperone protein specific for the TorA enzyme. It belongs to a family of TorD-like chaperones present in several bacteria, and, probably, involved in TMAO reductase folding.

Reduction of trimethylamine N-oxide (TMAO) in *Escherichia coli* involves the terminal molybdoenzyme TorA, located in the periplasm, and the membrane anchored c-type cytochrome TorC. In this study, the role of the TorD protein, encoded by the third gene of the *torCAD* operon, is investigated. Construction of a mutant, in which the *torD* gene is interrupted, showed that the absence of TorD protein leads to a two times decrease of the final amount of TorA enzyme. However, specific activity and biochemical properties of TorA enzyme were similar to those of the enzyme produced in the wild type. Excess of TorD protein restores the normal level of TorA enzyme, and also, leads to the appearance of a new cytoplasmic form of TorA on SDS-polyacrylamide gel electrophoresis under gentle conditions. This probably indicates a new folding state of the cytoplasmic TorA protein when TorD is overexpressed. BIACore techniques demonstrated direct specific interaction between the TorA and TorD proteins. This interaction was enhanced when TorA was previously unfolded by heating. Finally, as TorA is a molybdoenzyme, we demonstrated that TorD can interact with TorA before the molybdenum cofactor has been inserted. As TorD homologue encoding genes are found in various TMAO reductase species, it was proposed to be a chaperone protein specific for the TorA enzyme. It belongs to a family of TorD-like chaperones present in several bacteria, and, probably, involved in TMAO reductase folding.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Preparation of Extracts**—The *E. coli* strains used in this study are: MC4100 (araD139DlacIPOZYA-argFylacIPOZYA-argFlacIPOZYA-argFlacIPOZYA-argFlacIPOZYA-argF) U169 rpsl thi) and three derivatives LCB620 (torA::MudII1734) (4), LCB641 (torD::Tn10), and RK5208 (mobA207::Mucts) (10). The cells were grown anaerobically as described by Silvestro *et al.* (11). For induction of the TMAO reductase, TMAO was added until a final concentration of 10 mM is reached. When necessary, isopropyl-D-thio-β-D-galactopyranoside (IPTG; 2 mM) was added and antibiotics were used at the following final concentrations: ampicillin, 50 μg/ml; chloramphenicol, 25 μg/ml; and spectinomycin, 50 μg/ml. The cultures were incubated at 37 or 32 °C according to their genotype. The preparation of the different extracts as well as periplasm and spheroplast fractions were performed as described by Silvestro *et al.* (3).

**Purification of Proteins**—The purification of TMAO reductase was performed from the periplasmic fraction of MC4100 cells (initial activity: 11 μmol of TMAO reduced/min/mg of protein). The TMAO reductase was purified to homogeneity by DE52 ion exchange chromatography, Mono Q HR 16/10, and preparative electrophoresis (5.5% polyacrylamide). 1 mg of pure TMAO reductase was obtained with a specific activity of 250 μmol of TMAO reduced/min/mg of protein. The protein FA product of the mobA gene, was purified as described by Palmer *et al.* (12).

Enrichment in TorD protein was achieved as follows: the supernatant fraction (16 mg of proteins in 40 mM Tris-HCl, pH 7.6, 1 mM benzoamide-HCl) of strain LCB641/p*torD*, grown anaerobically in the presence of IPTG, was loaded on a Sephadex G-75 column. The presence of TorD (>60% of total proteins) was tested by SDS-PAGE. The band of the expected size for TorD was submitted to amino-terminal sequencing after an electrophoresis on polyvinylidene difluoride membrane. The enzymes share several properties: (i) they are all molybdooenzymes located in the periplasm of the bacterium and (ii) in each case, it has been proposed that a membrane-anchored pentaheme c-type cytochrome feeds electrons to the terminal enzyme (6, 7). In *E. coli*, this cytochrome, TorC, is encoded by *torC*, the first gene of the *torCAD* operon (4, 8). While the role of the TorC and TorA proteins is well documented, the role of the third protein, TorD, predicted in the *torCAD* operon is not (4). However, the presence of TorD homologue proteins, not only in *R. capsulatus* and *R. sphaeroides* Tor systems (6, 7), but also in *Shewanella* species² is intriguing and suggests a similar role for this protein in these systems. As TorD contains two small hydrophobic segments, at its amino and carboxyl ends, respectively, it was proposed to be a membranous b-type cytochrome involved in the electron transfer pathway for TMAO reduction (4, 9).

In this study, we show that TorD is not a membrane-bound protein but rather a cytoplasmic protein able to interact specifically with the unfolded TorA protein. We propose that TorD belongs to a chaperone family specific for certain molybdoproteins.
sequencing was performed using an Applied Biosystem apparatus.

**Analytical Procedures**—Benzy1 viologen (BV)-TMAO reductase activity was measured at 37 °C by a spectrophotometric technique (11) based on the oxidation of reduced benzy1 viologen at 600 nm coupled to the reduction of TMAO. TMAO reductase activity in polycrylamide gel was revealed as described above, except that methyl viologen was used, instead of benzy1 viologen.

Quinone-TMAO reductase activity assay was as described for nitrate reductase activity assay (13), except that menadion (menaquinone analog) was used instead of duroquinol. Oxidation of menadion by extract containing TMAO reductase was coupled to oxidation of NADH by the diaphorase, which catalyzes NADH-menaquinone oxidoreduction.

The amount of TMAO reductase antigen, present in the extracts, was determined by rocket immunoelectrophoresis (14) using a polyclonal antiserum specific for TMAO reductase (100 μl). SDS-PAGE was performed using 7.5% or 15% polycrylamide gels (15). The electrophoretic transfer of proteins to nitrocellulose or membranes and immunodetection with anti-TMAO reductase were carried out as described by Tawbin et al. (16). ECL-Western blotting system was used as recommended by the supplier (Amersham). Protein concentrations were estimated by the technique of Lowry et al. (17).

**Bioimaging Analysis**—The interaction between TorA and TorD proteins was investigated with a biomolecular interaction analysis biosensor based analytical system (BIAcore; Pharmacia). All experiments were performed at 25 °C. The TorD containing fraction was concentrated against 10 mM acetate buffer to reach pH 3. The protein was then immobilized on a sensor chip CM5 (Pharmacia biosensor) through amine coupling. The carboxylic acid groups of a dextran matrix were activated with 70 μl (10 μM/min) of a mixture of 0.2 M N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide hydrochloride and 0.05 M N-hydroxysuccinimide (18). TorD protein was injected during 8 min (10 μM/min), resulting in approximately 2000 resonance units of immobilized protein and the reaction was stopped by the injection of 70 μl (10 μM/min) of 1 M ethanolamine hydrochloride in order to transform the remaining active esters into amides. This procedure allows the TorD protein to be covalently coupled to the carboxyl dextran-modified gold surface via the exposed amino groups, TMAO reductase, bovine serum albumin, or β-galactosidase (0.4 mg/ml) were injected using a constant flow rate of 10 μl/min. When required, purified proteins were heated for denaturation during 10 min at 75 °C and then placed at 4 °C until injection under the same conditions. The sensogram study was performed using the biomolecular interaction analysis evaluation software (Pharmacia).

**Activation of the Inactive Tor Protein in mob Crude Cell Extracts**—The activation was performed with either a fixed amount of TMAO reductase apoenzyme of mob strain (40 mg/ml, 100 μl) and increasing quantities of purified FA protein (1 μg/ml) or, alternatively, with increasing amounts of TMAO reductase apoenzyme of mob strain and a fixed amount of the supernatant fraction of strain LCB620 (TorA−) grown anaerobically (31 mg/ml, 100 μl). The assay mixtures were incubated at 37°C for 60 min. The reaction was stopped by cooling the samples on ice.

**DNA Manipulations**—Standard procedures for plasmid preparation, restriction endonuclease digestions, agarose gel electrophoresis, DNA purification, and ligation were as described by Sambrook et al. (19). Transformations were performed by the method of Chung and Miller (20). Chromosomal DNA was prepared as described previously (4). PCR (polymerase chain reaction) amplifications were performed as described in Joulin et al. (21).

**Construction of Plasmids pTorAD, pTorD**—The TorA or TorD coding sequence was amplified by PCR using oligonucleotides Am1 or Dr1 and Db1 and chromosomal DNA preparation from MC4100 cells. The sequence of Am1 (5′-ACGGCAAATGAGAGGAAATATGAGACAATAAC-3′) corresponds to a MunI site located downstream from the TorA coding region and was used as the primer for PCR amplification. The resulting plasmid (pMD1) was introduced into strain MC4100 at 30 °C. The cells were then incubated at 43 °C to allow integration of the plasmid by homologous recombination into the chromosomal torD gene. Cells were grown at 30 °C during 48 h without antibiotics then plated on spectinomycin containing medium. 21 Spr Cms clones were selected. To ascertain the location of the insertion in the chromosomal torD gene, we performed PCR amplifications with chromosomal DNA of 5 clones as template and with a primer located either at the beginning of the torA sequence (Am1) or downstream from the insertion site (Db1), together with a primer (2) complementary to both extremities of the insert. In all cases, the insert region was amplified within the torA gene. The amplification of the plasmid was also checked in cells grown at 30 °C. One clone was retained and called LCB641.

### RESULTS AND DISCUSSION

**Effect of Absence of TorD on the Tor System**—In *vivo*, TMAO reduction by TorA can be measured using a quinone homologue (menadione or benzyl viologen (BV)) as electron donor. Reduction of TMAO with quinone involves all the components of the electron transfer chain from the membranous quinone pool to the periplasmic terminal reductase. As a result, this activity represents an electron transfer close to the *in vivo* mechanism of electron transfer from the quinone analog to menadione or benzyl viologen. In the present study, the TorD mutant strain rules out the possibility for the protein TorD to be a component of the electron transfer chain from the membranous quinone pool to the periplasmic terminal reductase. As a result, this activity will represent an electron transfer closer to the *in vivo* mechanism than the BV-TMAO reductase activity involving only the terminal enzyme and the artificial electron donor.

Quinone-TMAO reductase activity was performed on crude extract of strain LCB641 (TorD−) grown under inducing conditions. The results reported in Table I shows that a quinone-TMAO reductase electron transfer does occur in strain LCB641. This activity is half of that measured in the cognate wild type strain (Table I). When determined by immunoelectrophoresis, the amount of TorA protein in the TorD− strain is also decreased two times, compared with the wild type strain, indicating that the TMAO reductase specific activity is similar in both TorD− and wild type strains. The existence of a relevant quinone-TMAO reductase activity in the TorD− mutant strain rules out the possibility for the protein TorD to be a constituent of the electron transfer chain. This is in agreement with the production of TMAO in *vivo* by strain LCB641 during the growth in anaerobiosis in the presence of TMAO.

BV-TMAO reductase activity was measured on crude extract of strain LCB641 (TorD−). Table I shows that the total TMAO reductase activity in this strain is at least two times lower than in the wild type MC4100 strain. Here again, specific activity is equivalent for both strains.

70% of the total BV-TMAO reductase activity of strain LCB641 (TorD−) is recovered in the periplasmic fraction of the
cells as described previously for the wild type (3). As expected, the amount of TMAO reductase protein in the periplasm is two times lower than the wild type counterpart (Fig. 1). No differences are observed between the TorA protein, synthesized in the mutant, and the wild type strain with regard to the SDS-electrophoretic pattern (Fig. 1A) and molecular weight. The strains used were grown in the presence of IPTG. A, LCB641/pTorD membranous (well 1, 10 μg), cytoplasmic (well 2, 13.5 μg), and periplasmic (well 3, 10 μg) fractions were loaded on 12.5% SDS-PAGE. B, the soluble fraction (50 μg) of strains LCB620/pTorD (well 4) and LCB620 (well 5) were loaded on 15% SDS-polyacrylamide gel.

Fig. 2. Location of the TorD protein. After electrophoresis, gels were stained with Coomassie Blue. 21- and 30-kDa molecular mass markers are indicated on the gels. The arrows indicate the TorD position. The strains used were grown in the presence of IPTG. A, LCB641/pTorD membranous (well 1, 10 μg), cytoplasmic (well 2, 13.5 μg), and periplasmic (well 3, 10 μg) fractions were loaded on 12.5% SDS-PAGE. B, the soluble fraction (50 μg) of strains LCB620/pTorD (well 4) and LCB620 (well 5) were loaded on 15% SDS-polyacrylamide gel.

Quinone- or BV-TMAO reductase activity measured in crude extract of this strain represents at least 90% of the wild type activity (Table I). Moreover, as for the wild type strain, 70% of BV-TMAO reductase activity as well as 70% of TorA protein were found in the periplasmic fraction of LCB641/pTorD cells. Complementation of the mutated strain LCB641 (TorD<sup>−</sup>) by plasmid pTorD restores both TorA protein concentration and TMAO reductase activity to the wild type level.

TorD, Which Is Located in the Cytoplasm, Is Neither a Heme-binding Protein nor a Transcriptional Regulator—As the TorD sequence exhibits at least two hydrophobic segments at the amino and carboxyl ends of the protein, a membranous location has been proposed. To determine unambiguously in which compartment of the cell the TorD protein is located, the membranous, periplasmic, and cytoplasmic fractions of LCB641/pTorD cells overproducing the TorD protein were analyzed by SDS-polyacrylamide gel electrophoresis. Surprisingly, a thick protein band with an apparent molecular mass close to that calculated from the amino acid sequence (22.5 kDa) was detected in the cytoplasmic fraction of strain LCB641/pTorD.

Fig. 1. Effect of TorD on the periplasmic TorA protein. Panel A, periplasmic fractions (6 μg of protein) of strains LCB641 (TorD<sup>−</sup>) (lane 1) and MC4100 (lane 2) both grown anaerobically in the presence of TMAO, and LCB641/pTorD grown as the latter with IPTG added (lane 3), were analyzed by Western blot with antibodies specifically raised against purified TorA protein after loading on 7.5% SDS-PAGE. Panel B, same periplasmic fraction, strain MC4100 (1.8 μg, well 1), LCB641 (TorD<sup>−</sup>) (2.4 μg, well 2), and LCB641/pTorD (1.8 μg, well 3) were submitted to immunoelectrophoresis rocket on an agarose plate where 100 μl of TorA antibodies were previously added.

TABLE II

K<sub>m</sub> obtained with different electron acceptors catalyzed by TorA from MC4100 or LCB641 (TorD<sup>−</sup>) strains

| Compounds<sup>a</sup> | MC4100 | LCB641 |
|----------------------|--------|--------|
| TMAO                 | 0.30<sup>b</sup> | 0.37   |
| 4-Methylmorpholine-N-oxide | 0.16 | 0.18   |
| 2-Picoline-N-oxide   | 11     | 9.16   |
| 3-Picoline-N-oxide   | 2.97   | 3.32   |
| Dimethyl sulfoxide    | ND     | ND     |
| Tetramethylene sulfoxide | ND | ND     |

<sup>a</sup> Assays were performed in 100 mM phosphate buffer (pH 6.8) with a substrate concentration of 10 mM as described by Iobbi-Nivol et al. (24).

<sup>b</sup> K<sub>m</sub> values are expressed in mM.

<sup>c</sup> ND, activity values obtained were too low for a K<sub>m</sub> calculation.

<sup>d</sup> Activity values obtained were too low for a K<sub>m</sub> calculation.
when analyzed by both TMBZ staining of SDS-PAGE and low temperature spectroscopy (data not shown), and (iii) the physiological electron transfer from a menaquinone analogue to the terminal enzyme still occurs in the TorD-defective strain LCB641 (Table I).

As TorD is located in the cytoplasm, we have also investigated the possibility that TorD is a transcriptional regulator of the torCAD operon. β-Galactosidase activity was measured in strain LCB620 grown in anaerobiosis and in the presence of TMAO. In this strain harboring a chromosomal torA-lacZ fusion, the torD gene, which is the last gene of the torCAD operon, is probably not expressed. When plasmid pTorD was introduced in this strain and IPTG added to the growth medium, the β-galactosidase activity remained unchanged. In addition, when bandshift assays were performed using the torCAD promoter and the TorD protein partially purified, no retardation of the DNA fragment was observed (data not shown). This strongly suggests that TorD has no affinity for the promoter sequence of the torCAD operon.

Overproduction of TorD Protein Modifies Cytoplasmic TorA Electrophoretic Mobility—As described previously (3), 70% of TorA is located in the periplasm of the cell whereas only about 25% is found in the cytoplasmic fraction. Study of TorA electrophoretic pattern of a strain overproducing TorD was carried out loading non-heated samples on 7.5% SDS-polyacrylamide gel. These conditions allow the TMAO reductase activity to be revealed directly on the gel and makes the detection of different folding states of this protein possible. As shown on Fig. 3, retardation of the DNA fragment was observed (data not shown). This result clearly shows that TorD has an affinity for the entire cytoplasmic TorA protein.

Overproduction of TorD in a Molybdenum Cofactor Defective Strain, mobA, Enhances Apoenzyme TorA Stability—If TorD is a TorA-specific chaperone then a binding between TorD and TorA is expected at least when TorA is unfolded. Interaction between these two proteins should be detected using an apparatus dedicated to study protein-protein contact, the BIAcore system (see, for example, Ref. 25). For this purpose, partially purified TorD protein was immobilized on the dextran matrix (see “Experimental Procedures”), while TorA was used in either a native or unfolded conformation.

An increase in the amount of recovered resonance units was observed after the native TorA protein was injected (Fig. 4A, 2, time > 440 s). A stronger signal (2.5 times more efficient) was obtained when the TorA protein was previously heated (Fig. 4A, 1). When the pH was changed from 5 to 7, the response was similar but the signal amplitude was less important (data not shown). Identical experiments were performed using an equivalent fraction originating from cells lacking the TorD protein. None of the tested TorA samples presented a specific interaction with the activated matrix (see “Experimental Procedures”), while TorA was used in either a native or unfolded conformation.

Overproduction of TorD results in an unusual folding of the entire cytoplasmic TorA protein.

TorD Interacts Directly with TorA—In vivo effect of overproduction of TorD protein on TorA enzyme. 5 μg of unheated periplasm protein (lanes 1 and 2) and 50 μg of unheated cytoplasm protein (lanes 3 and 4) samples from strain MC4100 (lanes 1 and 3) and LCB641/pTorD (lanes 2 and 4) were loaded on SDS-PAGE 7.5%. Panel A, methyl viologen-TMAO reductase activity. Panel B, Western blot using serum anti-TorA as in Fig. 1A.

Overproduction of TorD in the cell leads to a modification of the cytoplasmic TorA electrophoretic pattern suggesting that the protein can exist in different active conformational states. We then propose that TorD is a specific chaperone for TorA, and, that
crosses the inner membrane by a Sec-independent mechanism (26). The question arises whether TorD is able to interact with a TorA protein devoid of molybdenum cofactor. The processing of synthesis, insertion, and maturation of the molybdenum cofactor is a complex pathway involving the products of five transcriptional units (the mo- genes). The last step of this process requires the presence of protein FA, the mobA gene product. It has been shown that the amount of TorA apoprotein present in mo- strains is 3–4 times lower compared with the quantity observed in a wild type strain (11). The plasmid pTorD was introduced in a mobA mutant in which all the molybdoenzymes are devoid of molybdenum cofactor. The amount of TorA apoprotein was estimated in a crude extract of the mob/pTorD strain by immunoelectrophoresis rocket. As shown on Fig. 5, the amount of TorA apoprotein is increased when the torD gene is overexpressed, and reaches the level of enzyme present in the wild type strain. We can therefore conclude that TorD acts on TorA before the insertion of the molybdenum cofactor into the apoprotein and, as a result, probably protects the TorA apoprotein against degradation.

**In Vitro Reactivation of TorA When the torD Gene Is Overexpressed**—Molybdooapoenzymes from a mobA strain can be activated in vitro in the presence of either the purified protein FA or a crude extract containing FA protein. Since TorD can interact with TorA independently of the presence of the molybdenum cofactor, we analyzed the effect of TorD overproduction in this reactivation system. When the purified FA or FA-containing supernatant fraction (FA ‘TorA-’ strain) was used to reactivate TorA apoprotein, activity was restored to the maximum value of 0.2–0.3 μmol of reduced TMAO/min/mg of protein (Fig. 6, A and B). The same experiment was performed using a mobA (FA-) pTorD strain instead of the mobA strain. The activity, in this case, was higher since the maximum value obtained was about 0.8–1 μmol/min/mg of protein, explained by the higher quantity of TorA in this strain (Fig. 6, A and B).

In the experiment described on Fig. 6C, the amount of TorD protein varies according to the amount of FA protein. Despite the increased quantity of TorD protein, the level of apoTorA activation is not enhanced. Accordingly, the effect of the overproduction of TorD is only observed when both TorD and apo-

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**Fig. 5. Effect of excess of TorD protein on the amount of apo-TorA in a mobA strain.** Immunoelectrophoresis rocket is performed as in Fig. 1B. Crude extracts of strains mobA (FA-) (well 1, 100 μg and well 2, 200 μg) and mobA/pTorD (well 3, 60 μg and well 4, 120 μg) were loaded.

**Fig. 6. Overexpression of TorD protein in mobA-deficient strain enhances in vitro reactivation of apoTorA.** A, the system of activation of TMAO reductase contains 200 μl of strain mobA (FA-) (40 mg/ml) (○) or strain mobA/pTorD (40 mg/ml) (●) and an increasing amount of purified FA protein (1 μg/μl). The systems were incubated 60 min at 37 °C, before BV-TMAO reductase activity was measured. B, activation system containing 100 μl of supernatant of LCB620 strain (31 mg/ml) and increasing quantity of the apoTorA containing strains mobA (○) and mobA/pTorD (●). C, TMAO reductase activity is measured in the activation systems which are performed with 200 μl of mobA strain (30 mg/ml) and increasing volume of LCB620 (30 mg/ml) (△) or LCB620/pTorD (30 mg/ml) (▲) supernatants.

**Fig. 7. Alignment of TorD-like protein sequences with E. coli TorD (EcTorD).** TorD-like proteins are: DmsB from R. sphaeroides (RsDmsB), TorD from R. capsulatus (RsTorD), TorD from Shewanella species (SsTorD), Orf 1044 from H. influenzae (Hi1044), and Orf Ycac from E. coli (EcYcac). Residues shown in dark gray and bold face are identical residues present in at least 50% of the aligned sequences. Residues shown in light gray and bold face are conserved residues (DEY, NQTSC, KRH, and WGAFPMVLI) present in more than 50% of the aligned sequences. * highlights the presence of a residue in more than 80% of the aligned sequences. The two underlined regions correspond to the most conserved regions.
TorA are produced in the same cell.

These results indicate that most of the TorA inactive apoprotein present in the mohA/pTorD strain can be activated. The presence of a high amount of TorD protein probably decreases the turnover of the inactive TorA enzyme. Therefore, we propose that TorD interacts with TorA in an early stage of the enzyme synthesis.

**TorD Is Part of a Chaperone Family**—When the TorD protein was first mentioned (4), no homologous protein could be found in data banks. Since the release of the sequences of proposed operons encoding the Me$_2$SO/TMAO reductases from *R. sphaeroides* and *R. capsulatus* (DmsA and DorA, respectively), a gene encoding TorD homologous protein (DmsB and DorD, 26–27% of identity with TorD, Fig. 7) was found. Recently, the TMAO reductase system in a *Shewanella* species was also shown to contain a TorD-like protein sharing 34% identity with *E. coli* TorD$^2$ (Fig. 7). Starting from the complete sequence of the *Hemophilus influenzae* genome, we also found a TorD homologous protein. The structural gene (Hi1044) is close to the dms operon encoding a membranous Me$_2$SO reductase enzyme. Interestingly, in *E. coli*, an open reading frame (EcYea) located downstream from the dms operon and transcribed in the opposite orientation could also encode a TorD homologue. These proteins share an equivalent size (from 199 to 226 amino acids) and show at least two highly conserved regions (position 121–136 and 161–192 of *E. coli* TorD). We propose that all these proteins are part of a chaperone family specific for Me$_2$SO/TMAO molybdoenzymes (27).

In the absence of TorD, the TorA protein presents wild type properties but the amount of the protein is decreased compared with the wild type strain. A tentative explanation is that TorA is capable to fold on its own but with a less efficient ratio. Alternatively, another chaperone, which could be either a general molecular chaperone or a specific chaperone (for example, EcYea), takes over the folding of TorA. Surprisingly, Moncey et al. (28) have recently proposed that *R. sphaeroides* DmsB protein possesses an essential role in the TMAO respiratory pathway. Experiments are in progress to confirm that the TorD homologues play the same role toward their cognate enzyme as that of the *E. coli* TorD protein.

The presence of specific chaperone for molybdoenzymes is probably widespread in bacteria, since a nitrate reductase chaperone has been recently described for *E. coli* (29, 30). Although the NarJ nitrate reductase chaperone shares similar size with TorD, no apparent homologies could be detected between the two primary structures.

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