Reconstitution of the Trimethylamine Oxide Reductase Regulatory Elements of *Shewanella oneidensis* in *Escherichia coli*

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Several bacteria can grow by using small organic compounds such as trimethylamine oxide (TMAO) as electron acceptors. In *Shewanella* species, the TMAO reductase respiratory system is encoded by the *torECAD* operon. We showed that production of the TMAO reductase of *S. oneidensis* was induced by TMAO and repressed by oxygen, and we noticed that a three-gene cluster (*torSTR*) encoding a complex two-component regulatory system was present downstream of the *torECAD* operon. We introduced the *torSTR* gene cluster into *Escherichia coli* and showed that this regulatory gene cluster is involved in TMAO induction of the *torE* promoter but plays no role in the oxygen control. The TorR response regulator was purified, and gel shift and footprinting experiments revealed that TorR binds to a single region located about 70 bases upstream of the transcription start site of the *tor* structural operon. By deletion analysis, we confirmed that the TorR operator site is required for induction of the *tor* structural promoter. As the TMAO regulatory system of *S. oneidensis* is homologous to that of *E. coli*, we investigated a possible complementation between the TMAO regulatory components of the two bacteria. Interestingly, TorS$_{ec}$, the TMAO sensor of *E. coli*, was able to transphosphorylate TorR$_{so}$, the TMAO response regulator of *S. oneidensis*.

Under anaerobic conditions, many bacteria can grow by using alternative terminal electron acceptors such as nitrate, nitrite, dimethyl sulfoxide, or trimethylamine oxide (TMAO) (4, 27). TMAO is a widespread low-molecular-mass constituent of marine invertebrates and fish, in which it provides protection against stresses such as high concentrations of urea or hydrostatic pressure (36, 37). Various distant species, including enteric, photosynthetic, and marine bacteria, can obtain energy by reduction of TMAO into volatile trimethylamine (3, 11).

TMAO respiratory systems have been widely studied at the molecular level in *Escherichia coli* and *Rhodobacter* and *Shewanella* species (8, 19, 21, 29, 33). A common feature of these systems is the presence of three conserved structural components: a periplasmic molybdenum-containing reductase, a pentahemic *c*-type cytochrome anchored to the membrane, and a reductase-specific chaperone. Although TMAO is mainly found in the marine environment, few molecular studies have been performed for the TMAO respiratory system of marine bacteria, and little is known about the TMAO reductase system of *Vibrio* species (8, 26).

In *Shewanella massilia,* the terminal reductase TorA is encoded by the third gene of the *torECAD* operon. *torC, torD,* and *torE* encode, respectively, TorC, a membrane-anchored pentahemic *c*-type cytochrome; TorD, a TorA-specific chaperone; and TorE, a small membranous protein homologous to the NapE protein of *Thiosphaera pantotropha* (8). NapE belongs to the nitrate reductase system, but the roles of NapE and TorE are not yet known (4).

In *E. coli*, in addition to the *torCAD* structural operon, the *tor* locus comprises two regulatory genes, *torS* and *torR*, which encode the TorS/TorR two-component regulatory system (15). TorS is the transmembrane sensor that detects the presence of TMAO in the medium, and TorR is the response regulator that, once phosphorylated by TorS, activates the *tor* operon by binding to three regulatory sites (2, 14, 31). A third regulatory gene, *torT*, encoding a periplasmic protein similar to the ribose-binding protein, is also found in the *tor* locus (16). TorT probably binds a still unknown inducer and then interacts with the periplasmic detector region of TorS to stimulate *tor* induction. In *Rhodobacter* species, a two-component regulatory system homologous to the TorS/TorR proteins of *E. coli* is found, but the *Rhodobacter* system contains no protein similar to TorT (22, 29, 34). In these organisms, the regulatory gene clusters are located upstream of the structural operons (15, 21, 34). In *S. massilia,* although the Tor system is induced by TMAO, no regulatory gene was found upstream of the *torECAD* operon (8).

Usually, full induction of the TMAO reductase operons occurs in anaerobiosis. In *Rhodobacter* species, the anaerobic control is mediated by FnrL, a general anaerobic regulator (22, 38), whereas, in *E. coli*, it is mediated neither by FNR nor by ArcA, another global anaerobic regulator (32). In *Shewanella oneidensis* (formerly *S. putrefaciens* MR1), EtrA, an FNR analog, does not seem to be involved in TMAO reductase gene expression (18).

In this study, we show that the *tor* structural operon of *S. oneidensis* is tightly controlled by TMAO and, to a lesser extent, by anaerobiosis. The TMAO regulatory proteins of *S. oneidensis* constitute a complex two-component system related

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
E. coli		
MC4100	*araD139* Δ(*lacIPOZYA-argF*)*U169 rpsL thi*	M. J. Casadaban
LCB621	MC4100 *torR49*::mini-Tn *10* (Tc^r)	32
BW26416	BW25113 Δ(*torSTRCAD*) Km^r	7
LCB436	Δ(*torSTRCAD*) Km^r derivative of MC4100	This work
S. oneidensis ATCC 700550	Wild type, formerly *S. putrefaciens* MR1	23
Plasmids		
pBAD24	Vector containing P$_{BAD}$ promoter with a pBR origin of replication	13
pBAD33	Vector containing P$_{BAD}$ promoter with a pACYC origin of replication	13
pGE593	Operon fusion vector with a pBR origin of replication	10
pR$_{SO}$1	*torR* sequence from *S. oneidensis* inserted into pBAD24	This work
pR$_{SO}$2	*torR* sequence from *S. oneidensis* inserted into pBAD33	This work
pPTor$_{so}$4	*torE* promoter (−481 to +217)^a from *S. oneidensis* cloned into pGE593	This work
pPTor$_{so}$7	*torE* promoter (−84 to +119)^a from *S. oneidensis* cloned into pGE593	This work
pPTor$_{so}$9	*torE* promoter (−60 to +119)^a from *S. oneidensis* cloned into pGE593	This work
pSTR$_{SO}$	*torSTR* sequence from *S. oneidensis* cloned into pBAD33	This work
pTR$_{SO}$	pSTR$_{SO}$ but with *torS* partly deleted	This work
pSR$_{SO}$	pSTR$_{SO}$ but with *torT* disrupted	This work
pS$_{SO}$	pSTR$_{SO}$ but with *torR* and part of *torT* deleted	This work

^{a} Nucleotide positions relative to the transcription start site of *torE*.

to that of *E. coli*, but *tor* operon induction requires binding of the response regulator to only one operator site.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. The *S. oneidensis* strain (23) was grown at 30°C in rich medium with 50 mM TMAO when necessary. To maintain selection for plasmids or for transductants in *E. coli*, ampicillin and chloramphenicol were added at 50 μg/ml and kanamycin at 10 μg/ml. *E. coli* was grown at 37°C in Luria broth medium supplemented with 10 mM TMAO and 0.001% arabinose when required. In order to limit *torR$_{so}$* expression from pTorR$_{so}$2, 0.05% glucose was added when indicated. *E. coli* strain LCB436 was constructed by P1 transduction of the deletion (Δ*torSTRCAD*)-insertion (Km^r) of strain BW26416 (7) into strain MC4100. The deletion of the *tor* locus in strain LCB436 was checked by PCR with appropriate primers.

DNA manipulations. DNA preparations, restriction endonuclease digestions, purifications, and ligations were carried out according to standard procedures (28). Transformations were performed as described by Chung and Miller (5).

Construction of plasmids. To create plasmid pR$_{so}$1, we employed PCR with *S. oneidensis* chromosomal DNA as the template to generate DNA fragments corresponding to the *torR$_{so}$* coding sequence with an upstream *Eco*RI and a downstream *Sma*I site. After enzymatic hydrolysis, the PCR product was cloned into the same sites of pBAD24 (13). To create plasmid pR$_{so}$2, pR$_{so}$1 was digested with *Bam*HI, and the resulting *torR$_{so}$*-containing fragment was subcloned into the same sites of pBAD33 (13). In these plasmids, *torR* is under the control of the arabinose-inducible promoter.

To create plasmid pSTR$_{so}$, we used PCR with *S. oneidensis* chromosomal DNA as the template to generate a *torSTR*-containing DNA fragment (from position −182 relative to the first nucleotide of the initiation codon of *torR* to the stop codon of *torS*) with an upstream *Xba*I and a downstream *Sma*I site. The PCR product was cloned into the same sites of pBAD33, and the orientation was P$_{BAD}$-*torRTS*. To create plasmid pTR$_{so}$, the *torS* sequence of plasmid pSTR$_{so}$ was partly deleted (from position +1899 relative to the initiation codon of *torS*) after *Pst*I digestion, followed by intramolecular ligation. To create plasmid pSR$_{so}$, the *Kpn*I site of the *torT* gene of plasmid pSTR$_{so}$ was cleaved and blunted with T4 DNA polymerase (blunting kit from Takara) before ligation, leading to a frameshift at position +582 relative to the initiation codon of *torT*. To create plasmid pS$_{so}$, *torR* and a part of *torT* were deleted from plasmid pSTR$_{so}$ after *Kpn*I digestion, followed by intramolecular ligation.

We amplified the *torE* promoter region by PCR from positions −481 to +217, −84 to +119, and −60 to +119 relative to the *torE* transcription start site to create plasmids pPTor$_{so}$4, pPTor$_{so}$7, and pPTor$_{so}$9, respectively. The DNA fragments were blunted and introduced into plasmid pGE593 (10), previously linearized by *Sma*I, thus placing the *lacZ* coding sequence under the control of the putative *torE* promoter. Except for the large insert of plasmid pSTR$_{so}$, all the cloned fragments and fusion sites were confirmed by sequencing. To minimize the number of spontaneous mutations, the *torSTR$_{so}$* gene cluster was amplified with only 20 cycles, and three independent pSTR$_{so}$ clones were tested.

Analytical procedure. Crude extracts of *S. oneidensis* were made as previously described for *E. coli* (30). The periplasmic fraction was prepared by the procedure of Easter et al. (9). The periplasmic proteins were analyzed by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (SDS–7.5% PAGE). After electrophoresis, TMAO reductase was visualized either directly by an activity staining method (25) or by immunodetection with antibodies against TorA of *S. massilia* after protein transfer to a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane. The ECL-Western blotting system was used as described by the supplier (Amersham Pharmacia Biotech). The protein concentrations were estimated by the technique of Lowry et al. (17).

Primer extension analysis. Strain LCB436 carrying pPTor$_{so}$4 and pR$_{so}$2 and the *S. oneidensis* strain were grown anaerobically in the presence of TMAO until the culture reached an A_{600} of 0.5. Total RNA was prepared by using the Qiagen RNA extraction kit. The oligonucleotide 5′-AAGAGTATGAAAATGATGAA TCCCAG-3′, complementary to a *torE* coding region, was end labeled with [γ-^{33}P]ATP (2,500 Ci/mmol) by using T4 polynucleotide kinase and purified with the Qiagen Qiaquick nucleotide removal kit. The primer extension reaction was performed with reverse transcriptase (Superscript II from Gibco-BRL). The sequencing ladder was generated with the same oligonucleotide used for the primer extensions.

β-Galactosidase assays. β-Galactosidase activities were measured on whole cells by the method of Miller (20) either after overnight anaerobic growth or after aerobic growth performed in rotating Erlenmeyer flasks when the culture reached 0.4 to 0.5 A_{600} unit. Values represent the averages of at least three independent experiments with a variation of no more than 15% from the mean.

Purification of TorR and TorS proteins. Overproduction of the TorR$_{so}$ protein was achieved by growing 100 ml of strain MC4100 carrying plasmid pR$_{so}$1. When the culture reached an A_{600} of 1, overproduction of the TorR protein was induced for 1 h with 0.2% arabinose. The cells were then harvested by centrifugation, and the pellet was resuspended in 5 ml of 40 mM Tris-HCl, pH 7.6. After disruption of the cells by French press, the extract was centrifuged at 14,000 rpm for 10 min. The supernatant was directly loaded on a heparin-Sepharose column (Pharmacia). The proteins were eluted with a step gradient of KCl from 100 mM to 1 M. TorR$_{so}$ was purified to near homogeneity in the 400 mM KCl fraction. The *E. coli* TorR and TorS726 proteins were purified as described previously (14, 32).

Preparation of labeled DNA fragments. A 295-bp and a 271-bp DNA fragment encompassing the *tor* regulatory region were generated by PCR from pPTor$_{so}$7 and pPTor$_{so}$9 with appropriate labeled and unlabeled primers. Labeling was carried out with [γ-^{32}P]ATP (4,000 Ci/mmol) and T4 polynucleotide kinase
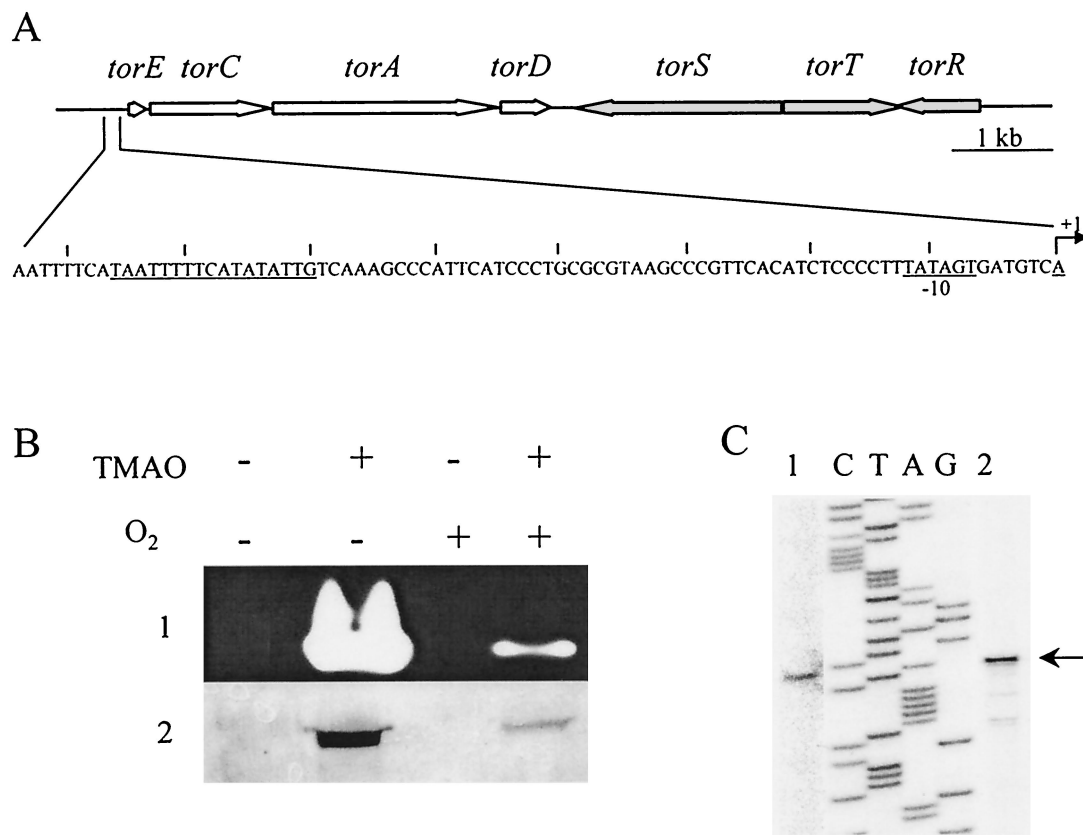
FIG. 1. (A) Physical map of the *tor* locus of *S. oneidensis*. The large arrows show the locations and orientations of the *tor* genes. The nucleotide sequence of the *torECAD* regulatory region is indicated. The +1 arrow corresponds to the transcription start site. The putative −10 promoter box and the TorR binding region are underlined. (B) SDS-PAGE analysis of TMAO reductase production of *S. oneidensis* grown anaerobically (−O$_2$) or aerobically (+O$_2$), in the presence (+) or absence (−) of TMAO. Nonheated (row 1) or heated (row 2) periplasmic extracts (15 μg) were loaded on the gel. After electrophoresis, the presence of TorA$_{so}$ was checked by staining the gel for TMAO reductase activity (row 1) or by a Western blot with anti-TorA$_{sm}$ antibodies (row 2). (C) Localization of the transcription start site of *torE*. A labeled primer complementary to *torE* internal sequence was annealed to total RNA from *S. oneidensis* cells grown anaerobically in the presence of TMAO (lane 1) or to RNA from *E. coli* LCB436 (pPTor4-pR$_{so}$2) cells grown anaerobically in the presence of 0.001% arabinose (lane 2), and extended with reverse transcriptase. The primer extension reactions were loaded on each side of a sequencing ladder of the *torE* region made with the same primer as in the extension reaction.

(Gibco-BRL), and the labeled fragments were separated from unincorporated nucleotides by using a column of the nucleotide removal kit from Qiagen.

Gel retardation assays. Binding of TorR to labeled DNA fragments (7 nM) was carried out in a 4-μl reaction mixture containing 50 mM Tris-HCl (pH 8), 1.25 mM EDTA, 0.25 M sucrose, 0.025% bromophenol blue, and 0.25 μg of poly(dI-dC)·poly(dI-dC) per μl. After 30 min at room temperature, samples were loaded and run on a native 12.5% polyacrylamide gel (Pharmacia Phast System). The gel was exposed for 3 h at room temperature.

DNase I footprinting. The footprinting experiments were performed as follows. The labeled DNA fragment was diluted to a concentration of about 1 nM in 50 μl of binding mix [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2.5 mM MgCl$_2$, 0.5 mM dithiothreitol, 4% glycerol, and 30 ng of poly(dI-dC)·poly(dI-dC) per μl], and different amounts of TorR were then added. After 30 min of incubation at room temperature, DNase I was added (0.33 U), and the reaction was conducted for 1 min, then stopped by the addition of 140 μl of DNase stop solution (192 mM sodium acetate, 32 mM EDTA, 0.14% SDS, and 64 μg of yeast RNA per ml). After phenol-chloroform extraction and DNA-ethanol precipitation, the pellets were resuspended in loading solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and loaded on a 6% polyacrylamide–8.3 M urea electrophoresis gel. The location of the protected nucleotides was deduced by running a ladder with the products of the G+A cleavage reaction.

Phosphorylation assays. For the phosphorylation experiments, proteins TorS726, TorR$_{so}$, and TorR$_{ec}$ (5 μM each) were incubated 10 min with 0.1 mM

[γ-^{32}P]-ATP (1 μCi) in 10 μl of buffer containing 50 mM Tris-HCl (pH 7.6), 4 mM dithiothreitol (DTT), 0.5 mM EDTA, 5 mM MgCl$_2$, 10% glycerol, and 60 mM KCl. The reactions were stopped by addition of 5 μl of loading buffer (200 mM Tris-HCl [pH 8.8], 5 mM EDTA, 1 M sucrose, 0.1% bromophenol blue, 6% SDS, 0.1 M DTT, 1.6% β-mercaptoethanol) and incubated at 55°C for 3 min. The samples were analyzed after SDS-PAGE with the Pharmacia Phastsystem apparatus.

RESULTS AND DISCUSSION

Induction of TMAO reductase in *S. oneidensis*. During a structural analysis of the periplasmic TMAO reductase terminal enzyme (TorA$_{sm}$) of *S. massilia*, we also sequenced the gene encoding the TMAO reductase (TorA$_{so}$) of *S. oneidensis* strain MR1 (formerly *S. putrefaciens* MR1) (6, 35). Starting from this sequence, we retrieved the surrounding DNA sequence of *torA$_{so}$* from the website of the Institute for Genomic Research (http://www.tigr.org). Analysis of the sequence revealed that *torA$_{so}$* belongs to a four-gene cluster (Fig. 1A) homologous to the *torECAD* operon of *S. massilia*, and the
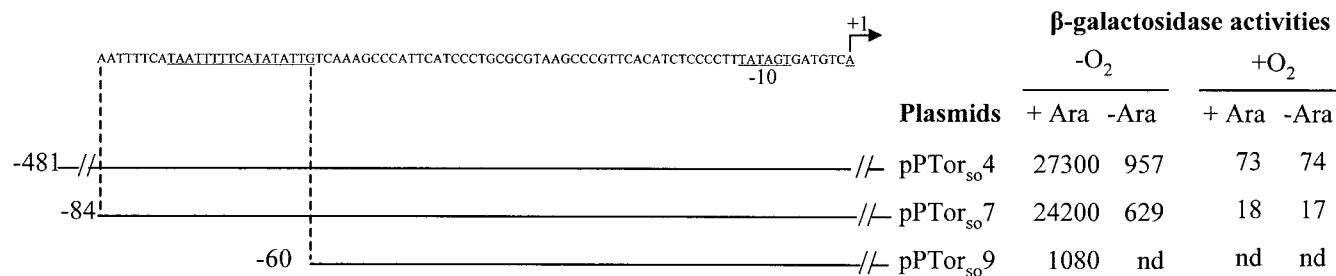
FIG. 2. Effect of TorR$_{so}$ overproduction and of deletions in the *torE* promoter region on *tor-lacZ* fusion expression in *E. coli*. The TorR binding site and the putative −10 box are underlined, and the transcription start point of *torE* is indicated by the arrow at +1. The 5′ end of the cloned *tor* sequence is shown, whereas the 3′ part of the *tor* region is not shown and corresponds to position +217 for pPTor$_{so}$4 and +119 for pPTor$_{so}$7 and pPTor$_{so}$9, relative to the *torE* transcription start site. Strain LCB436 containing plasmid pR$_{so}$2 (*torR$_{so}$*) and a plasmid of the pPTor$_{so}$ series, as indicated, was grown anaerobically (−O$_2$) or aerobically (+O$_2$), in the presence (+) or absence (−) of 0.001% arabinose. β-Galactosidase activities of the plasmid-borne *tor-lacZ* fusions are expressed in Miller units. nd, not determined.

gene products show 83, 98, 93, and 70% identity with TorE, TorC, TorA, and TorD of *S. massilia*, respectively.

To characterize the pattern of expression of the putative *torECAD* operon of *S. oneidensis*, we examined the inducing conditions for the TorA$_{so}$ enzyme. Periplasmic extracts were prepared from *S. oneidensis* cells grown aerobically or anaerobically in the presence or absence of TMAO, and TMAO reductase activity was detected after loading nonheated samples on an SDS-polyacrylamide gel (Fig. 1B). Maximum activity was observed for the strain grown anaerobically with TMAO, and the mobility of the active band corresponded to that expected for TorA (about 90 kDa). The presence of oxygen during growth strongly reduced the activity level, and no activity was detected in the absence of TMAO.

To confirm that the TMAO reductase active bands corresponded to TorA enzyme, we carried out a Western blot from an equivalent gel with antibodies against TorA$_{sm}$. TorA$_{sm}$ and TorA$_{so}$ are highly homologous, and the anti-TorA$_{sm}$ antibodies recognized TorA$_{so}$ (Fig. 1B). Antibody binding to TorA$_{so}$ confirmed that the intensity of the TMAO reductase active bands reflects the amount of TorA enzyme. Strict control by the substrate and, to a lesser extent, by anaerobiosis appears to be a general regulatory feature of TMAO respiratory systems, since the same inducing conditions have been observed in various bacteria (8, 15, 22, 26). However, the *cis* and *trans* elements involved in the regulation of *Shewanella* species have not yet been characterized.

To define the promoter of the *torECAD* operon of *S. oneidensis*, we carried out a primer extension experiment using RNA prepared from cells grown anaerobically with TMAO (Fig. 1C). The transcription start site was located 33 bases upstream of the *torE* start codon, and a −10 promoter box (TATAGT) close to the *E. coli* −10 consensus sequence is correctly positioned relative to the start site. In contrast, no putative −35 box matching the *E. coli* consensus sequence could be found 16 to 18 bp upstream of the TATA box (Fig. 1A). The absence of a −35 consensus box supports the idea that the *S. oneidensis* tor promoter is positively regulated and that transcription initiation requires binding of an activator. In *E. coli*, TorR, the activator of the *tor* operon, binds to four decameric direct repeats (31), but no such repeats could be found upstream of the TATA box of the *S. oneidensis* tor

promoter. Moreover, no sequence related to the *E. coli* FNR box (TTGATNNNNATCAA) was seen in the *tor* regulatory region, in agreement with the fact that EtrA, the *S. oneidensis* analog of the pleiotropic anaerobic regulator FNR, plays no role in the regulation of the Tor system (18).

Activation of *S. oneidensis* tor promoter by overproduction of TorR response regulator. In contrast to the *E. coli* tor locus (32), no gene encoding a response regulator is present upstream of the *tor* structural genes in *S. oneidensis*. However, three genes located just downstream of the *torECAD* operon and named *torS*, *torT* and *torR* might encode the regulatory proteins of the Tor system (Fig. 1A). Indeed, *torS* encodes a putative transmembrane protein of 1,010 amino acids homologous to the TorS sensor of *E. coli* (32% identity), while *torR* and *torT* encode a response regulator and a periplasmic protein showing 55 and 34% identity with the TorR response regulator and the TorT regulatory protein of *E. coli*, respectively.

As genetic tools for *S. oneidensis* are poorly developed, we decided to investigate the regulation of the *torECAD* promoter in *E. coli*. To avoid interference between the Tor systems of the two strains, we used an *E. coli* strain (LCB436) deleted of the entire *tor* locus. A DNA fragment containing *torE* and the untranscribed region upstream of *torE* was fused to the *lacZ* coding sequence of the operon fusion vector pGE593, and the *torR$_{so}$* coding sequence was cloned downstream of the arabinose-inducible P$_{BAD}$ promoter of plasmid pBAD33. The two plasmids (pPTor$_{so}$4 and pR$_{so}$2), which are compatible, were introduced together into strain LCB436, and the effect of TorR overproduction on expression of the *torEC'-lacZ* fusion was followed.

As shown in Fig. 2, in anaerobiosis, the β-galactosidase activity of the fusion increased strongly when arabinose was added. Overproduction of TorR thus activates expression of the *torEC'-lacZ* fusion. As overproduction of response regulators has often been employed to mimic regulator activation by phosphorylation (24, 32), this result strongly suggests that TorR is the response regulator responsible for induction of the *torECAD* operon. However, it does not prove that the same promoter sequence is used in *E. coli* and in *S. oneidensis*. To clarify this point, we carried out a primer extension experiment to determine the transcription start site of the *torEC'-lacZ*

A

Complex →

Free DNA →

1 2 3 4 5 6 7 8 9 10 11 12

B

G+A   1 2 3 4 5 6 7

- 60—

-76—

C

So AAAATTTTCATAATTTTTCATATATTGTCAAAGCCCATTCATCCCTGCGCGTAAGCCCGTTCACATCTCCCCTTTATAGTGATGTCA

Ec ATTCTGTTCATATCTGTTCATATTCTGCCGTAAGCCGTTCATCCTGACCAGTGCCGCTGTTCATATTTGCTCATTAAGATCGCTTCA
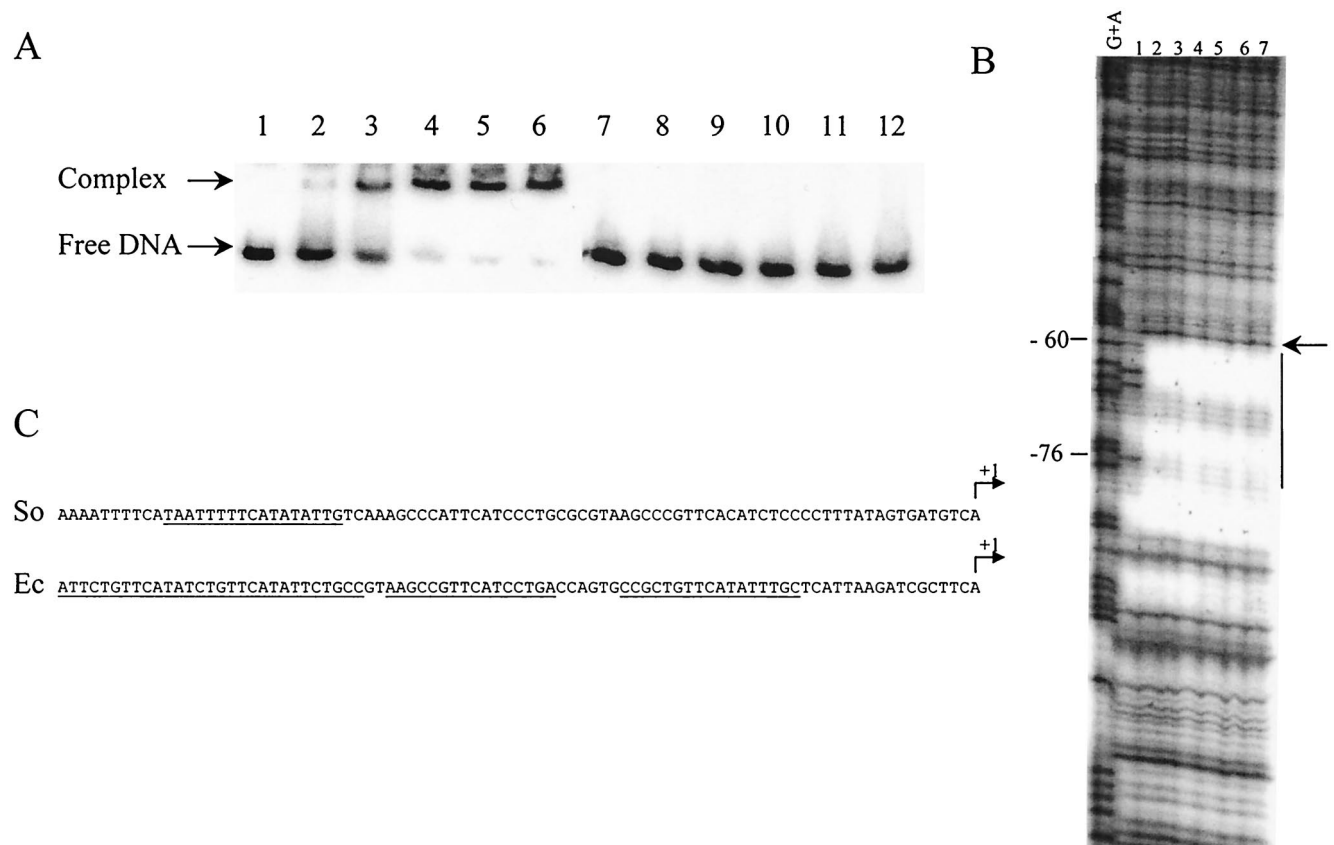
FIG. 3. Analysis of TorR binding to the *torE* promoter region by band shift assays (A) and DNase I footprinting experiments (B). (A) Labeled DNA fragments containing the *torE* region from positions −84 to +119 (lanes 1 to 6) or from −60 to +119 (lanes 7 to 12) were incubated in the presence of the following concentrations of purified TorR protein: lanes 1 and 7, no protein; lanes 2 and 8, 0.05 μM; lanes 3 and 9, 0.25 μM; lanes 4 and 10, 0.5 μM; lanes 5 and 11, 1 μM; and lanes 6 and 12, 2.5 μM. (B) The labeled DNA fragment was prepared by PCR using sense ^{32}P-end-labeled and reverse unlabeled primers and plasmid pPTor$_{so}$7 as the template. The fragment was digested with 0.33 U of DNase I in the presence of various concentrations of purified TorR$_{so}$ protein: lane 1, no protein; lane 2, 0.25 μM; lane 3, 0.5 μM; lane 4, 1 μM; lane 5, 2.5 μM; lane 6, 5 μM; and lane 7, 10 μM. The G+A sequencing ladder is indicated. Numbering is relative to the transcription start site. Vertical bar indicates the protected region, and the arrow shows a DNase I-hypersensitive site. (C) Nucleotide sequences of the *S. oneidensis* (So) and *E. coli* (Ec) *tor* promoters. The regions protected by TorR$_{so}$ or TorR$_{ec}$ are underlined, and transcription start sites are indicated by arrows labeled +1.

fusion (Fig. 1C). RNA was prepared from the strain containing both plasmids grown anaerobically with arabinose, and the primer used was complementary to the *torE* coding sequence. The same transcription start site was used in *E. coli* and *S. oneidensis*, indicating that transcription initiates from the same promoter in both bacteria.

Location of TorR binding region in *tor* operon promoter of *S. oneidensis*. To confirm that TorR induces expression of the *tor* operon directly by binding to the *tor* promoter, we purified TorR and carried out a DNA-binding gel shift assay (Fig. 3A). When the DNA fragment contained the *tor* region from positions −84 to +119 relative to the transcription initiation site, a retarded band was observed in the presence of small amounts of TorR and the apparent equilibrium binding constant, K_{eq}, was about 250 nM. Increasing the concentration of TorR led to retardation of most of the labeled DNA, but the same retarded band appeared even at the highest TorR concentration, suggesting that TorR binds only one DNA site in the *tor* promoter.

We identified the TorR binding region by DNase I footprinting analysis. A 295-bp DNA fragment containing the *tor* pro-

moter (from positions −84 to +119) was generated by PCR using one labeled oligonucleotide and incubated with increasing concentration (0 to 10 μM) of TorR. Only one region of about 17 bp was protected even at the highest TorR concentrations (Fig. 3B). A DNase I-hypersensitive site appeared at position −59, suggesting that increased exposure of the minor groove to DNase I attack resulted from DNA bending induced by TorR binding. The protected region is A/T rich but contains neither palindromic structures nor direct repeats (Fig. 3C). Almost the same region was protected against DNase I when the DNA fragment was labeled on the opposite strand or when TorR was preincubated with 100 mM acetyl phosphate or carbamoyl phosphate, two small phosphodonor molecules for response regulators (data not shown). These results suggest that TorR activates the *torECAD* operon by binding to a single site centered at position −68 and contrast with the situation observed for the *torCAD* promoter of *E. coli*, in which three distinct regions were protected by TorR$_{ec}$ (31) (Fig. 3C).

To test whether sequences upstream of the TorR binding site affected promoter function, we amplified a DNA fragment

| | | | | β-galactosidase activities (*torE'-lacZ*) | | | |
| | | | | -O$_2$ | | +O$_2$ | |
			Plasmids	+ TMAO	-TMAO	+ TMAO	-TMAO
torS	*torT*	*torR*	pSTR$_{so}$	12500	2100	2850	111
			pTR$_{so}$	1900	1620	44	38
			pSR$_{so}$	1550	2510	30	28
			pS$_{so}$	47	31	nd	nd
			No	50	47	nd	nd

FIG. 4. Effect of the *torS*, *torT*, and *torR* genes of *S. oneidensis* on *torE'-lacZ* fusion expression in *E. coli*. The arrows show the locations and orientations of the cloned genes, and disruption and deletions of the reading frames are also indicated. Strain LCB436 containing pPTor$_{so}$7 and plasmid pSTR$_{so}$ or a pSTR$_{so}$ derivative, as indicated, was grown anaerobically ($-O_2$) or aerobically ($+O_2$), in the presence (+) or absence (−) of TMAO. β-Galactosidase activities are expressed in Miller units. nd, not determined.

carrying the *tor* promoter sequence from positions −84 to +119 and cloned it upstream of the *lacZ* reporter gene of plasmid pGE593 to generate pPTor$_{so}$7. The β-galactosidase activity measured when TorR was overproduced in anaerobiosis indicated that the *tor* promoter starting just a few bases upstream of the TorR box was expressed almost as strongly as that starting 400 bases further upstream (Fig. 2). In contrast, deletion of the TorR box (pPTor$_{so}$9) resulted in both a >20-fold decrease in activity (Fig. 2) and complete absence of DNA retardation (Fig. 3A). The TorR operator site is thus essential for *tor* operon expression, whereas sequences upstream of position −84 are dispensable.

Involvement of *torS* and *torT* in TMAO control of *tor* operon expression. To determine whether *torS* and *torT*, the two genes located between *torD* and *torR* (Fig. 1A), play a role in *tor* operon regulation, we amplified the putative regulatory gene cluster *torSTR* by PCR and cloned it into pBAD33. The resulting plasmid, pSTR$_{so}$, was introduced together with pPTor$_{so}$7 into strain LCB436, and expression of the plasmid-borne *torE'-lacZ* fusion was monitored in various growth conditions (Fig. 4). Strikingly, addition of TMAO led to a strong increase in the fusion activity in both aerobiosis and anaerobiosis. The *torSTR* gene cluster might thus be involved in TMAO induction of the *tor* operon. The *torE'-lacZ* fusion was no longer induced by TMAO when *torS* or *torT* was inactivated. In anaerobiosis, the significant activity levels measured in the absence of TorS or TorT probably came from expression of the multicopy *torR* gene, which was cloned with its promoter sequence. In the absence of TorR, the activity of the fusion dropped to the background level, confirming that TorR is essential for induction of the *tor* operon.

Together these results support the idea that TorS, TorT, and TorR constitute the TMAO regulatory system responsible for *tor* operon expression. As this probable TMAO regulatory system comprises three components homologous to those of the TMAO phosphorelay system of *E. coli* (1, 14), we propose a similar mechanism of signal transduction in which sensor TorS detects both TorT and TMAO and transphosphorylates response regulator TorR via a four-step phosphorelay. However, although the TorS sensors of both strains contain three phosphorylation sites, their N-terminal detector regions show no similarity (data not shown). TMAO detection as well as TorT interaction could thus implicate unrelated detector sites.

In *E. coli*, neither FNR, ArcB, nor TorR plays the role of the Tor anaerobic regulator (32). In *S. oneidensis*, the Tor anaerobic control does not seem to involve EtrA (18), the FNR homologue, and TorR appears to be implicated in the TMAO regulatory pathway. Surprisingly, when expressed in *E. coli*, the *tor* operon promoter of *S. oneidensis* was still under the control of anaerobiosis (Fig. 2 and 4). The TMAO regulatory proteins seem to play no role in this control, since anaerobic regulation still occurred in the absence of TorS or TorT, but the function of TorR$_{so}$ itself might be oxygen labile. Another possibility is that this anaerobic control involves essential proteins highly conserved among these strains. Alternatively, DNA supercoiling could play a role in the *tor* anaerobic control of both strains.

Phosphotransfer from the *E. coli* TorS sensor to the *S. oneidensis* TorR response regulator. As the TorR proteins of *S. oneidensis* and *E. coli* are homologous (62.5 and 52% identity for the receiver and DNA-binding domains, respectively), we tested whether *E. coli* TorR could activate the *tor* operon of *S. oneidensis*. To answer this question, we introduced pPTor$_{so}$7 (*torE'-lacZ*) into an *E. coli* strain containing the wild-type chromosomal *tor* locus. The activity from the fusion remained very low when TMAO was added (Fig. 5A). The phosphorylated form of *E. coli* TorR (TorR-P) was thus incapable of activating the *tor* operon promoter of *S. oneidensis*. This is not surprising, because the TorR binding site of the *S. oneidensis* *tor* promoter is different from that of the *E. coli* *tor* operon promoter (Fig. 3C). Therefore, TorR-P from *E. coli* was probably unable to bind the *tor* promoter of *S. oneidensis*. Functional complementation of *torT$_{so}$* by the TorT protein of *E. coli* was also unsuccessful (data not shown).

Although the *E. coli* TorR and TorT proteins cannot substitute for the *S. oneidensis* homologs, we wondered whether the TorS sensor of *E. coli* could replace that of *S. oneidensis*. Plasmids pR$_{so}$2 and pPTor$_{so}$7 were introduced together into a

A

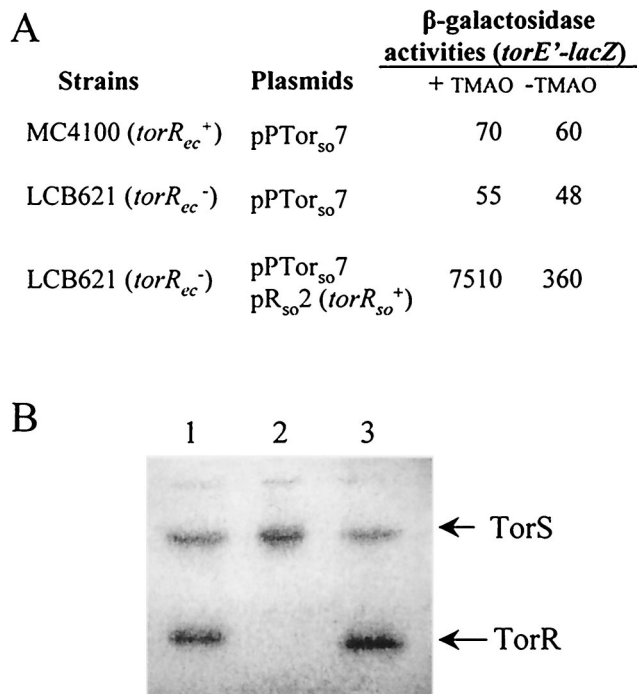| Strains	Plasmids	β-galactosidase activities (*torE'-lacZ*)	
		+ TMAO	-TMAO
MC4100 ($torR_{ec}^+$)	pPTor$_{so}$7	70	60
LCB621 ($torR_{ec}^-$)	pPTor$_{so}$7	55	48
LCB621 ($torR_{ec}^-$)	pPTor$_{so}$7 pR$_{so}$2 ($torR_{so}^+$)	7510	360

B

FIG. 5. Signal transduction from TorS of *E. coli* to TorR of *S. oneidensis*. (A) Effect of *E. coli* and *S. oneidensis* response regulator TorR on *torE'-lacZ* fusion expression in *E. coli*. Strain MC4100 ($torR_{ec}^+$) carrying pPTor$_{so}$7 (*torE'-lacZ*) and strain LCB621 ($torR_{ec}^-$) carrying pPTor$_{so}$7 alone or with pR$_{so}$2 ($torR_{so}^+$) were grown anaerobically in LB medium supplemented with glucose to limit $torR_{so}$ expression, in the presence (+) or absence (−) of TMAO. β-Galactosidase activities of the plasmid-borne *torE'-lacZ* fusion are expressed in Miller units. (B) Autophosphorylation of the *E. coli* TorS726 sensor and transphosphorylation of the TorR response regulators from *S. oneidensis* and *E. coli*. TorS726 alone (lane 2) or with TorR$_{so}$ (lane 1) or TorR$_{ec}$ (lane 3) was incubated at room temperature for 10 min in the presence of [γ-^{32}P]ATP, and the reaction mixtures were analyzed on SDS–20% PAGE. The autoradiogram of the gel is shown. The positions on the gel of the TorR and TorS726 proteins are indicated.

torS$^+$ *torR* *E. coli* strain, and the activity of the *torE'-lacZ* fusion was measured in cells producing a small amount of TorR$_{so}$. The activity of the fusion was low without TMAO but strongly increased in the presence of TMAO (Fig. 5A). This result suggests that the *E. coli* TorS sensor can transphosphorylate the *S. oneidensis* TorR response regulator, although the histidine phosphotransfer domain of *E. coli* TorS has only 26.3% identity with that of *S. oneidensis*.

To show that *E. coli* TorS can directly transphosphorylate *S. oneidensis* TorR, we performed an in vitro experiment with mutant TorS726, a constitutively active *E. coli* TorS protein (14), and purified *S. oneidensis* TorR. In the presence of [γ-^{32}P]ATP, TorS726 was labeled, and after addition of TorR$_{so}$, part of the TorS radioactivity was transferred to TorR (Fig. 5B). The transfer was quite efficient compared to the control experiment with TorR from *E. coli*. This biochemical analysis thus confirms the genetic data and shows that *E. coli* TorS can activate *S. oneidensis* TorR by transphosphorylation. The C-terminal domain of TorS, which is the phosphodonor site for its cognate response regulator, probably does not discriminate between the two TorR proteins.

In conclusion, the *S. oneidensis torSTR* gene cluster encodes a regulatory system involved in induction of the *tor* structural operon in response to TMAO. This TMAO regulatory system might regulate expression of additional genes. In particular, the gene encoding the soluble fumarate reductase of a *Shewanella* species is strongly downregulated by TMAO (12), and TorR might play the role of a repressor in this case.

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