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The *torR* Gene of *Escherichia coli* Encodes a Response Regulator Protein Involved in the Expression of the Trimethylamine *N*-Oxide Reductase Genes

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Expression of the *Escherichia coli torCAD* operon encoding the trimethylamine *N*-oxide (TMAO) reductase system is induced by both TMAO and anaerobiosis. A *torR* insertion mutant unable to express the *torA* gene had previously been isolated. The *torR* gene was cloned and sequenced. It encodes a 25,000-Da protein which shares homology with response regulators of two-component systems and belongs to the OmpR-PhoB subclass. Overproduction of TorR mimicks the presence of the inducer TMAO while the anaerobic control is unchanged, suggesting that TorR mediates only the TMAO induction. The overproduced TorR protein was purified to more than 90%. The *torR* gene is located just upstream of the *torCAD* operon, with an opposite transcription direction. The *torR-torCAD* intergenic region is unusual in that it contains four direct repeats of a 10-nucleotide motif. Part or all of these motifs could be involved in the binding of TorR. The gene encoding the sensor partner does not seem to be adjacent to *torR*, since the divergent open reading frame found immediately downstream of *torR* exhibits none of the features of a protein histidine kinase.

Enterobacteria can use a variety of terminal electron acceptors, including oxygen, nitrate, trimethylamine *N*-oxide (TMAO), dimethyl sulfoxide (DMSO), or fumarate. The reduction of TMAO by *Escherichia coli*, which allows anaerobic growth on nonfermentable sources, such as glycerol (2), is due mainly to the TMAO reductase pathway, with a small contribution from the DMSO reductase complex (32). The TMAO reductase is a monomeric molybdoprotein located in the periplasm (31, 32). Recently, Méjean et al. (18) identified and sequenced *torA*, the structural gene of TMAO reductase. It belongs to the *torCAD* operon, located at 22 min on the chromosome, in which *torC* codes for a membrane-bound *c*-type cytochrome with five heme-binding sites, whereas the function of the product of *torD* is still unknown (18).

The transcription of the *torCAD* operon is subject to at least two kinds of regulation. The first is anaerobiosis (24): shifting from aerobic to anaerobic conditions results in a 10-fold induction of *torA* expression. Remarkably, in contrast to all other known anaerobic respiratory systems, this control is mediated neither by the Fnr protein, the general anaerobic regulator (33), nor by the ArcA protein (14, 26). The second type of regulation is the induction by TMAO and related compounds, such as DMSO and tetrahydrothiofene (24, 32); this control is so strict that no expression is detected in the absence of an inducer.

When *E. coli* is grown in the presence of nitrate, the activated NarL protein induces the *narGHJI* (nitrate reductase) operon but represses the *dmsABC* (DMSO reductase) and *frdABCD* (fumarate reductase) operons (10, 13). This repression is thought to allow the cells to preferentially use the most energetically favorable substrate (13). Even though the redox potential of the TMAO-trimethylamine couple is far lower than that of the NO_3^--NO_2^- couple (+130 versus +420 mV), the expression of the *torCAD* operon is not subjected to this hierarchical control of nitrate (24).

Previously, Pascal et al. (25) reported the isolation of a *torR* insertion mutant affected in the expression of the *torA* gene and proposed that *torR* encodes a positive regulator. This mutation, which does not seem to affect the synthesis of the TMAO-DMSO reductase encoded by the *dmsABC* genes, is located at 22 min and strongly linked to *torA* (18). In this paper, we present genetic, molecular, and biochemical characterizations of TorR. Collectively, these studies permit us to propose that TorR mediates the TMAO induction.

MATERIALS AND METHODS

Strains and growth conditions. Bacteria and plasmids are listed in Table 1. Bacteria were usually grown on L-broth medium (19). For the β-galactosidase assays, cells were grown on minimal medium (4) supplemented with glucose (0.2%) and TMAO (4 mM) when necessary. Tetracycline and kanamycin were used at the concentration of 25 μg ml^{-1}; ampicillin was used at the concentration of 50 μg ml^{-1}. The concentration of isopropyl-β-D-thiogalactopyranoside (IPTG) was 1 mM.

DNA techniques. Plasmid preparations, restrictions, and ligations were carried out by standard procedures (28). The chromosomal DNA preparation and PCR techniques have been described elsewhere (18). DNA fragments were purified from agarose gels by using GeneClean (Bio 101). Transformations were performed by the method of Chung and Miller (7).

Determination of the miniTn*10* insertion sites. The chromosomal insertion site of miniTn*10* was determined by inverse PCR (21). Chromosomal DNA of the strain under investigation was digested by appropriate restriction endonucleases and ligated under dilute conditions (1 μg ml^{-1}) in order to favor intramolecular circularization. PCR amplification was performed on this ligated DNA, using as primers two outward-facing oligonucleotides in the 3′ region downstream of gene

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TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or phenotype	Origin
Strains		
MC4100	*araD139* Δ(*lacIPOZYA-argF*)*U169 rpsL thi*	M. J. Casadaban
LCB620	MC4100 *torA8*::MudII1734	M. C. Pascal
LCB621	MC4100 *torR49*::miniTn*10*	25
LCB640	LCB620 with *pcnB* and *zad*::Tn*10*	This work
620-621	LCB620 with *torR49*::miniTn*10*	25
Plasmids		
pLM4 and pLM5	Bluescript KS$^+$ and KS$^-$ with 3.8-kb *Bgl*II-*Bam*HI inserts from pMCP1	18
pGS1	*torR* sequence inserted into pJF119EH	This work

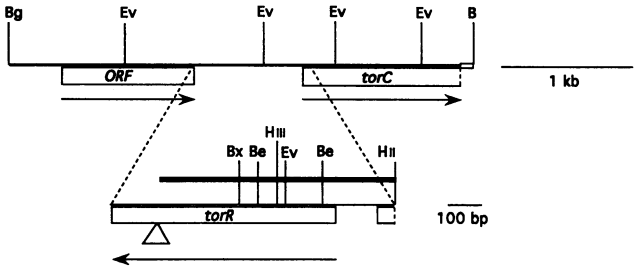

FIG. 1. Physical map of the *Bam*HI-*Bgl*II fragment upstream of the *torCAD* operon. The 117-bp segment containing the *Bam*HI site (open bar) belongs to the MudII4042 plasmid, which was used to construct a *torA-lac* fusion (18). The ORFs (rectangles) and their transcription directions (arrows) are indicated. The *torR* region is enlarged and its restriction map is more complete. The thick line above *torR* represents the material amplified by inverse PCR from strain LCB621, with the miniTn*10* insertion indicated by a triangle. Restriction endonucleases: B, *Bam*HI; Be, *Bst*EII; Bg, *Bgl*II; Bx, *Bst*XI; Ev, *Eco*RV; HII, *Hae*II; HIII, *Hae*III.

tetA (30) whose sequences were 5′-TCACCAACGCTTTTC CCGAGA and 5′-AAACGCCATTGTCAGCAAATTGA.

DNA sequence determination. Inserts in Bluescript KS$^+$ and KS$^-$ were shortened by exonuclease III (3). Single-stranded DNA was sequenced by the dideoxy chain termination method (29). For direct sequencing of PCR fragments, the reaction was performed with Sequenase as previously described (27).

Primer extension analysis. Strain MC4100 was grown anaerobically to late exponential phase. Total RNA was prepared by the hot phenol method (19). A synthetic oligonucleotide complementary to a sequence on the *torR* coding region (positions 2001 to 2026 from the *Bgl*II site) was end labelled with [γ-^{32}P]ATP by T4 polynucleotide kinase and was coprecipitated with 20 μg of RNA. The primer extension reaction was performed with reverse transcriptase by the method of Ausubel et al. (1) except that Moloney murine leukemia virus reverse transcriptase was used at 42°C for 90 min in the procedure. The sequencing ladder was generated by using DNA template corresponding to the mRNA sequence and the same oligonucleotide used for primer extension.

Construction of plasmid pGS1. The *torR* sequence was amplified by PCR using oligonucleotides R1 and R2 as primers. The sequence of R1 (5′-AGAATTCAAGGAGCCCTCT GATGCCACATCA) is composed of a *Eco*RI recognition site followed by a strong ribosome-binding site (AAGGAG) and of 18 nucleotides corresponding to the beginning of the *torR* sequence. The sequence of R2 (5′-ATGGATCCTAGCACA CATCAGCGGCTAAG) contains a *Bam*HI cleavage site followed by a triplet complementary to the TAG stop codon and by 19 nucleotides complementary to the very 3′ end of the *torR* coding sequence. The *Eco*RI-*Bam*HI fragment was introduced into the polylinker cloning sites of plasmid pJF119EH (12) to give plasmid pGS1.

Purification of the TorR protein. Overproduction of TorR was achieved by growing 100 ml of strain LCB620 carrying plasmid pGS1. When the culture reached 1 A_{600} unit, production of the TorR protein was induced for 1 h with 1 mM IPTG. The cells were then harvested by centrifugation, and the pellet was gently resuspended in 5 ml of buffer A (50 mM Tris-HCl [pH 7], 1 mM EDTA, 1 mM β-mercaptoethanol). After disruption of the cells by sonication, the extract was centrifuged at 12,000 × *g* for 10 min and the pellet was discarded. The supernatant was directly loaded on a 1-ml heparin-Sepharose column (Pharmacia) equilibrated with buffer A. The column was washed with 5 ml of buffer A and eluted with 0.5-ml fractions of 200 mM, 400 mM, 600 mM, 800 mM, and 1 M KCl. The TorR protein was eluted in the 600 mM fraction. This fraction was dialyzed against distilled water. The protein concentration was determined by the method of Bradford (5), using the Bio-Rad protein assay kit system (Bio-Rad Laboratories). Amino acid sequencing of a dried protein fraction was performed with an automated amino acid sequencer (Applied Biosystems model 473A).

Gel electrophoresis. Samples to be analyzed were added to a 1/2 volume of loading buffer (100 mM Tris-HCl [pH 8], 2.5 mM EDTA, 500 mM sucrose, 0.005% bromophenol blue, 6% sodium dodecyl sulfate [SDS], 100 mM dithiothreitol, 1 mM β-mercaptoethanol), and the mixture was heated to 95°C for 10 min and stored at −20°C. Proteins were separated on an SDS–20% polyacrylamide gel (Pharmacia PhastSystem) and stained with Coomassie brilliant blue (PhastSystem Development).

β-Galactosidase assays. β-Galactosidase activities were measured for whole cells by the method of Miller (19); values are averages of at least triplicate determinations.

Nucleotide sequence accession number. The nucleotide sequence has been submitted to the GenBank EMBL Data Bank with the accession number X78195.

RESULTS

Position of the miniTn*10* insertion in strain affected in *torA* expression. Strain LCB621 is unable to express the *torA* gene as a result of an insertion of miniTn*10* in the *torR* gene (25). To determine the sequence of the inactivated gene as well as the insertion site of miniTn*10*, inverse PCR experiments were performed. For this purpose, the chromosomal DNA of this strain was digested with *Eco*RV, *Hae*II, or *Hae*III, ligated, and amplified with divergent oligonucleotides internal to the miniTn*10* (30, 37). The amplified fragments were subsequently analyzed with different restriction enzymes. Given the positions of the restriction sites in miniTn*10* (37), a map of the adjacent region was deduced from the size of the amplified fragments. This map matches perfectly that of the region immediately upstream of *torC* (Fig. 1), with a distance between the miniTn*10* insertion point and the beginning of *torC* of ca. 700 bp. The insertion site was then accurately determined by direct sequencing of an amplified fragment using the oligonucleotide internal to the miniTn*10*.

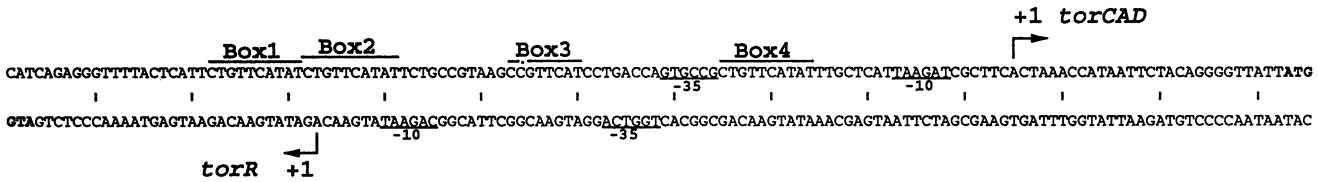

FIG. 2. Nucleotide sequence of the *torCAD* and *torR* regulatory region. The transcription start sites of *torR* and *torCAD* (+1 arrows), the putative −10 and −35 (underlining), the four identical boxes (overlining), and the translation start sites of *torC* and *torR* (boldface) are indicated.

miniTn*10* insertion in strain LCB621 disrupts a response regulator gene. Plasmids pLM4 and pLM5 (18), which carry a 3.8-kb insert of the analyzed region, were shortened by unidirectional degradation with exonuclease III, and the region between *torC* and the *Bgl*II site (Fig. 1) was sequenced on both strands.

The sequence analysis of the 2,248-bp fragment extending upstream of the beginning of *torC* revealed the presence of an open reading frame (ORF) starting with ATG at position 2119 from the *Bgl*II site and extending to TGA at position 1430. This ORF is disrupted at position 1566 by the miniTn*10* insertion; therefore, it corresponds to the *torR* gene under investigation. It is oriented divergently relative to *torC*, and the distance between the two translation start sites is 129 nucleotides (Fig. 2). The ATG is preceded by a noncanonical ribosome-binding site, GTAAAA. It encodes a putative protein of 230 residues with a calculated molecular mass of 25,000 Da. The amino acid sequence was compared with those of the proteins listed in the Swissprot data bank. Significant similarity with response regulators of the two-component regulatory system was detected (23, 35). Greater similarity (Fig. 3) was found with ArcA (43% identity), OmpR (32% identity), and PhoB (29% identity). The N-terminal part displayed similarity with the effector domain of these proteins, which contains the most-conserved amino acids that correspond to Asp-12, Asp-55, and Lys-105 of OmpR (23). Further similarities extending through the C-

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                                  *
TorR    1      M P H H I V I V E D E P V T Q A R L Q S Y F T Q G G Y T V S V T A
ArcA    1    M Q T P H I L I V E D E L V T R N T L K S I F E A E G Y D V F E A T
OmpR    1  M Q E N Y K I L V V D D D M R L R A L L E R Y L T E Q G F Q V R S V A
PhoB    1      M A R R I L V V E D E A P I R E M V C F V L E Q N G F Q P V E A E

                                                  *
TorR   34  S G A G L R E I M Q N Q S V D L I L L D I N L P D E N G L M L T R A L
ArcA   35  D G A E M H Q I L S E Y D I N L V I M D I N L P G K N G L L L A R E L
OmpR   36  N A E Q M D R L L T R E S F H L M V L D L M L P G E D G L S I C R R L
PhoB   34  D Y D S A V N Q L N E P W P D L I L L D W M L P G G S G I Q F I K H L

TorR   69  R E R S - - - T V G I I L V T G R S D R I D R I V G L E M G A D D Y V
ArcA   70  R E Q A - - - N V A L M F L T G R D N E V D K I L G L E I G A D D Y I
OmpR   71  R S Q S N - - P M P I I M V T A K G E E V D R I V G L E I G A D D Y I
PhoB   69  K R E S M T R D I P V V M L T A R G E E E D R V R G L E T G A D D Y I

                *
TorR  101  T K P L E L R E L V V R V K N L L W R - I D L A R Q A Q P H T Q D N L
ArcA  102  T K P F N P R E L T I R A R N L L S R T M N L G T V S E E R R S V E S
OmpR  104  P K P F N P R E L V L R I R A V L R R Q A N E L P G A P S Q E E A V I
PhoB  104  T K P F S P K E L V A R I K A V M R R - - - - - - I S P M A V E E V I

TorR  135  Y R F A G Y C L N V - S R H T L E R D G E P I K L T R A E Y E M L V A
ArcA  137  Y K F N G W E L D I N S R S L I G P D G E Q Y K L P R S E F R A M L H
OmpR  139  A F G K F K - - L N L G T R E M F R E D E P M P L T S G E F A V L K A
PhoB  133  E M Q G L S - - L D P T S H R V M A G E E P L E M G P T E F K L L H F

TorR  169  F I T N P G E I L S R E R L L R M L S A R R V E N P D L R T V D V L I
ArcA  172  F C E N P G K I Q S R A E L L K K M T G R E L K P H D - R T V D V T I
OmpR  172  L V S H P R E P L S R D K L M N L A R G R E Y S A M E - R S I D V Q I
PhoB  166  F M T H P E R V Y S R E Q L L N H V W G T N V Y V E D - R T V D V H I

TorR  204  R R L R H K L - - - - - S A D L L V T Q H R E G Y F L A A D V C
ArcA  206  R R I R K H F E S T P D T P E I I A T I H G E G Y R F C G D L E D
OmpR  206  S R L R R M V E E D P A H P R Y I Q T V W G L G Y V F V P D G S K A
PhoB  200  R R L R K A L E P - G G H D R M V Q T V R G T G Y R F S T R F
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FIG. 3. Sequence similarities between TorR and other regulators: ArcA (11), OmpR (9), and PhoB (17). Identical residues at a given position are indicated in boldface. The three amino acids highly conserved in regulators (asterisks) are indicated.

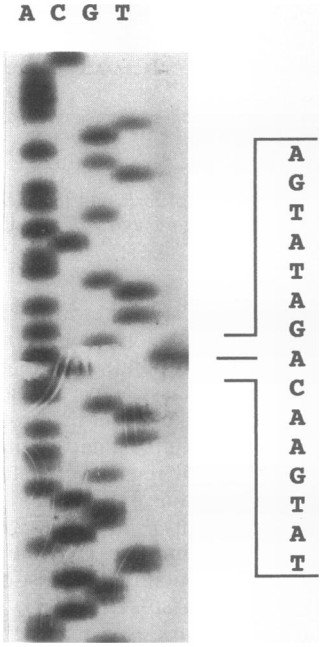

FIG. 4. Transcription start point of *torR*. The result of a primer extension reaction was analyzed by a comparison with an adjoining sequencing ladder. The sequencing reactions were done with the same primer as in the primer extension reaction.

terminal part assigned TorR to the PhoB-OmpR subclass, which regulates transcription of specific target genes at promoters which are thought to be recognized by the σ^{70} RNA polymerase holoenzyme.

Another ORF, divergent with *torR*, starts at position 425 from the *Bgl*II site and terminates at position 1452 from this same site (Fig. 1). Its 3′ end overlaps 22 nucleotides of the *torR* gene. The putative protein, which displays no significant homology with any protein listed in the Swissprot, Genpep, or Pir data bank, does not contain any amino acid motifs which are characteristic of histidine kinases.

Localization of the transcription start site of the *torR* gene. The transcription start site of *torR* was determined by using RNA prepared from wild-type cells grown anaerobically (Fig. 4). Extension of a primer complementary to the *torR* coding sequence indicated that the transcription is initiated at position 2149. As the *torCAD* transcription start site has been previously located at position −28 relative to the translation start site of *torC* (18), the untranscribed region between the two transcription units is only 71 bp long (Fig. 2). This short distance implies that the putative −35 sequences of the two promoters are back-to-back.

Overproduction and purification of the TorR protein. The *torR* coding sequence was amplified by the PCR technique; to optimize TorR production, a consensus ribosome-binding sequence, AAGGAG, was substituted for the natural one. The amplified fragment was then cloned downstream from the IPTG-inducible *tac* promoter of plasmid pJF119EH to give plasmid pGS1. To ensure that no mutation was introduced during amplification, the sequence of the cloned fragment was verified directly by DNA sequencing.

Strain LCB620 harboring plasmid pGS1 was grown aerobically in rich medium in the presence of IPTG. When the soluble cell lysate was analyzed by SDS-polyacrylamide gel electrophoresis, a thick protein band was detected (Fig. 5, lane 3), with an apparent molecular mass (27,000 Da) which was in good agreement with that calculated from the amino acid sequence of TorR. This band was absent in the strain grown without IPTG in the medium (Fig. 5, lane 1) as well as a strain harboring plasmid pJF119EH without the insert (Fig. 5, lane 2).

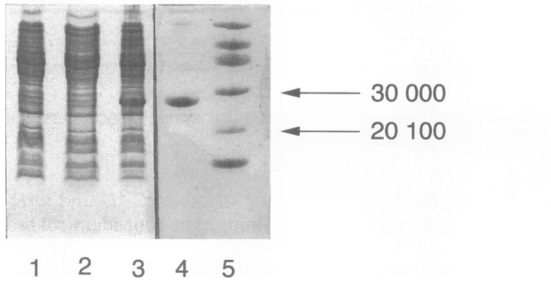

FIG. 5. Characterization and purification of the TorR protein. Lane 1, extract from LCB620/pGS1 grown without IPTG; lane 2, extract from LCB620/pJF119EH grown with IPTG; lane 3, extract from LCB620/pGS1 grown with IPTG; lane 4, purified TorR after heparin-Sepharose chromatography of the sonicated extract of LCB620/pGS1 grown with IPTG; lane 5, molecular weight standards with sizes indicated.

TorR was purified in one step by heparin-Sepharose chromatography. A sample of crude extract obtained by sonication of cells of strain LCB620/pGS1 was applied to a heparin-Sepharose column, and the TorR protein was eluted around 600 mM KCl with more than 90% purity (Fig. 5, lane 4). This simple purification technique produced 2.5 mg of purified TorR protein from 100 ml of culture.

The N-terminal amino acid sequence of the purified protein (Met-Pro-His-His-Ile-Val-Ile) was identical to that deduced from the nucleotide sequence of *torR*, unambiguously identifying the overproduced protein as the TorR protein.

Effect of in vivo overproduction of TorR on *torA-lac* fusion expression. Plasmid pGS1 was introduced into the *torR torA-lac* double mutant (strain 620-621), and the β-galactosidase activity of the *torA-lac* fusion was determined under various growth conditions (Fig. 6). Cells grown anaerobically in the absence of TMAO exhibited a high activity which was increased only twofold upon addition of TMAO. Cells grown aerobically in the presence or in the absence of TMAO displayed activities 8- and 25-fold lower, respectively, than those of the cells grown anaerobically. These experiments were carried out in the absence of IPTG since it proved to be toxic when added to the cells.

In order to examine the correlation between the amount of TorR protein per cell and the level of β-galactosidase activity observed, plasmid pGS1 was introduced into the *pcnB torA-lac* strain LCB640. Because of the *pcnB* mutation (16), the average *torR* copy number was very low. In this strain, the regulation of the *torA-lac* fusion was similar to that of the wild-type strain: the presence of TMAO and anaerobic conditions were both required for the expression of the fusion, as observed for strain LCB620. Upon IPTG induction of *torR* synthesis, the fusion was expressed in the absence of TMAO to a high level which was only slightly increased by the addition of TMAO. Once again, anaerobiosis was required for full induction, as was found in the high-copy-number plasmid experiments (Fig. 6).

Taken together, these results indicate the following. (i) Overproduction of TorR rendered TMAO dispensable for the expression of the *torA-lac* fusion. This overproduction can be achieved either by leakage of the *tac* promoter from plasmid

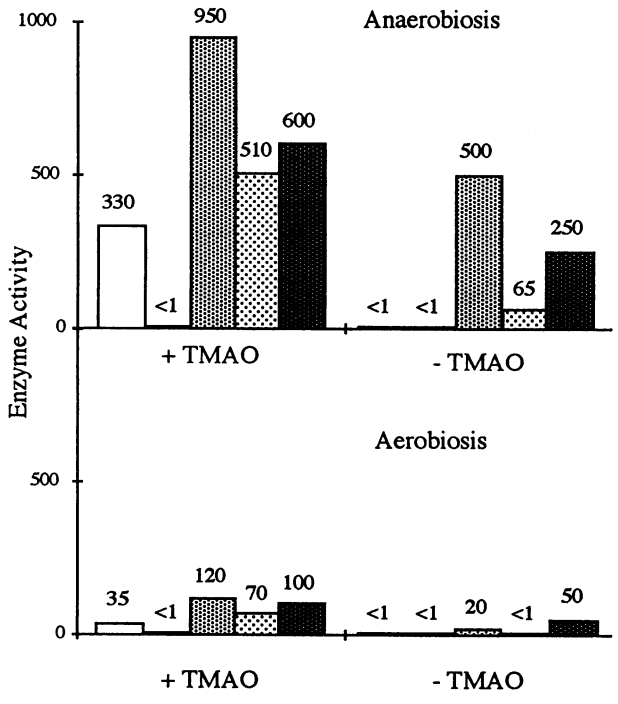

FIG. 6. β-Galactosidase activities of *torA-lac* fusion strains carrying plasmid pGS1, grown anaerobically or aerobically, with or without TMAO. Activities are expressed in Miller units (19). □, LCB 620; ■, 620-621 (*torR*); ▩, 620-621/pGS1; ▨, LCB 640 (*pcnB*)/pGS1; ■, LCB 640/pGS plus IPTG.

pGS1 at a high copy number or by IPTG induction of plasmid pGS1 at a low copy number. (ii) TorR overproduction does not supplant the need for anaerobic induction, since anaerobiosis is still required for *torA-lac* expression.

DISCUSSION

Previous genetic studies led us to propose that TorR is a positive transcriptional regulator for the expression of *torA*, which encodes TMAO reductase (25). In this study, the *torR* gene has been cloned and sequenced. Alignment analysis showed that the TorR protein exhibits all the features characteristic of a response regulator belonging to a two-component system such as those found in many prokaryotes (35) and some eukaryotes (6, 22).

Two-component regulatory systems consist of at least two proteins, including a sensor, or histidine protein kinase, and a response regulator (23, 35). Upon detection of an environmental signal, the histidine kinase autophosphorylates and the phosphate group is subsequently transferred to the response regulator. As the induction of the *torCAD* operon requires both anaerobic conditions and the presence of TMAO, TorR could be involved in either of these controls or both. Its strong similarity (43% identity) with ArcA, the general aerobic regulator (14), first suggested that TorR could mediate the anaerobic regulation. Production of TorR to a high level invalidated this hypothesis, since it led to *torA-lac* expression in the absence of TMAO but left the anaerobic control unchanged. The first conclusion to be drawn is that the two regulation mechanisms operating on the *torCAD* control region, i.e., TMAO induction and anaerobic control, are clearly distinct. The second point is that when TorR is present at a high enough concentration, it can bind its target DNA and activate *torCAD* transcription. This activation results from the binding of either an unphosphorylated form of TorR or a TorR protein phosphorylated by nonpartner sensors (36). We propose that TorR is the mediator of the TMAO induction, but isolation and characterization of *torR* constitutive mutants will be necessary to confirm this hypothesis.

The *torR* gene is located at 22 min immediately upstream from the *torCAD* operon and is transcribed in the opposite direction. Since genes for partner sensors and response regulators are often contiguous and since many of them are organized in operons (36), we have sequenced the DNA adjacent to *torR*. However, the divergent ORF found downstream does not contain any apparent amino acid motifs specific for histidine protein kinases and therefore, unless it belongs to an as-yet-undescribed class, does not seem to correspond to a sensor.

In an attempt to obtain other regulatory mutants, miniTn*10* insertions leading to the lack of *torA* expression have been isolated, but the only negative regulatory mutants obtained until now affect the *torR* gene (data not shown). Although sensor genes are generally much longer than regulator genes, no sensor mutant could be obtained, suggesting an essential role for this sensor gene. Another possible explanation for this negative result is that the TorR protein could be phosphorylated by two different protein histidine kinases, as is the case for the NarL protein, which can be phosphorylated either by NarX or by NarQ (34). If this is the case for TorR, disclosure of one of its sensor partners might be achieved by the isolation of a constitutive mutant.

Primer extension analysis identified the transcription start site of *torR* at a 71-bp distance from the transcription start site of *torCAD* (Fig. 2). Two hexanucleotides, TGGTCA and CAGAAT, that match four of the six bases in the −35 (TTGACA) and −10 (TATAAT) consensus sequences, respectively, for the σ^{70} promoter were located with appropriate spacings upstream of the transcription start site of *torR*, the −35 sequence being adjacent to the putative −35 sequence of the *torCAD* promoter. The absence of Fnr and NarL consensus sequences (15, 33) upstream of *torR* is consistent with the lack of regulation of the *torCAD* operon by these two proteins (24). No palindromic structure could be detected in this untranscribed DNA region, but a puzzling feature is the presence of four boxes of 10 nucleotides (CTGTTCATAT) in direct repeats (Fig. 2). Box 1 and box 2 overlap the transcribed *torR* region, from +12 to −8 from the transcription start point. Box 3, which matches only seven of the 10 bases, lies between the −10 and −35 sequences of the *torR* promoter, while box 4 lies between the −10 and −35 sequences of the *torCAD* promoter. Except in this *tor* regulatory region, this 10-nucleotide motif is present only four times in the known DNA sequence of *E. coli*, and two adjacent boxes were never found. Therefore, the presence of four boxes, three of which are identical, within 70 nucleotides should be highly significant. An attractive hypothesis is that they constitute the binding sites for a regulatory protein, an obvious candidate being TorR itself. The simple purification of TorR should allow testing of this hypothesis. On the other hand, the four boxes could also be the binding sites for a possible anaerobic regulator. This regulator could be either an aerobic repressor which competes with TorR for the binding sites or an anaerobic activator which associates with TorR to allow *torA* transcription (8). Alternatively, the effect of aerobiosis could be explained as a secondary consequence of changes in DNA topology (20) or inactivation of the TorR protein.

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