JOURNAL OF BACTERIOLOGY, Feb. 1996, p. 1219–1223 Vol. 178, No. 4
0021-9193/96/$04.00+0

The Periplasmic TorT Protein Is Required for Trimethylamine *N*-Oxide Reductase Gene Induction in *Escherichia coli*

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Received 26 September 1995/Accepted 2 December 1995

Expression of the *Escherichia coli torCAD* operon, which encodes the trimethylamine *N*-oxide reductase system, is regulated by the presence of trimethylamine *N*-oxide through the action of the TorR response regulator. We have identified an additional gene, *torT*, located just downstream from the *torR* gene, which is necessary for *torCAD* structural operon expression. Insertion within the *torT* gene dramatically reduced the expression of a *torA′-′lacZ* fusion, while presence of the gene in *trans* restored the wild-type phenotype. Overproduction of TorR in a *torT* strain resulted in partial constitutive expression of the *torA′-′lacZ* fusion, suggesting that TorR acts downstream from TorT. The *torT* gene codes for a 35.7-kDa periplasmic protein which presents some homology with the periplasmic ribose-binding protein of *E. coli*. We discuss the possible role of TorT as an inducer-binding protein involved in signal transduction of the *tor* regulatory pathway.

Escherichia coli is a facultative anaerobe which can use trimethylamine *N*-oxide (TMAO) as a terminal electron acceptor in the absence of oxygen (3). The main pathway responsible for reduction of TMAO to trimethylamine requires the *torCAD* operon, which is composed of three genes encoding a membrane-associated *c*-type cytochrome (TorC), a periplasmic molybdoenzyme (TorA), and a third protein (TorD) of unknown function (11, 13). The expression of the *tor* operon is tightly controlled by two environmental signals, i.e., the presence of TMAO in the medium and anaerobiosis (19, 28). TorR, the regulator responsible for TMAO induction of *tor* operon expression, has recently been identified as a member of the two-component regulatory system family (20, 28). Like other response regulators, TorR acts as an activator element, binding directly to a *cis*-acting regulatory region upstream from the structural gene coding sequences and presumably triggering transcription from the target promoter (12, 27). It now seems established that response regulators are activated by a transphosphorylation reaction (5, 18, 29). Upon detection of TMAO, an undescribed histidine kinase sensor, tentatively called TorS, would autophosphorylate and transfer its phosphate to its TorR partner, which in turn would activate transcription of the *tor* structural genes.

In the two-component regulatory systems, the kinase and regulator genes are often physically adjacent on the chromosome (8, 16, 31). However, a sensor or regulator gene alone was found in some cases in *E. coli* (16, 31). Immediately downstream from *torR* and convergently transcribed, there is an open reading frame which, if translated, would encode a polypeptide sharing no homology with the classical histidine kinases of two-component transduction systems (28). The fact that this open reading frame, designated *torT*, overlaps *torR* by 28 nucleotides (Fig. 1) prompted us to determine whether the putative TorT polypeptide plays any role in *tor* gene regulation. Our results show that the *torT* gene encodes a periplasmic protein involved in the expression of the *tor* operon.

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Characterization of the *torT* gene. In the course of determining the DNA sequence of the *torR* region (28), a second open reading frame was found just downstream and in the opposite orientation of *torR* (Fig. 1). On the basis of the start codon at position 432 and the stop codon at position 1459 relative to the nearest *Bgl*II site, this open reading frame is 1,026 nucleotides in length and would encode a polypeptide composed of 342 amino acid residues. Furthermore, the ATG initiation codon is preceded by a potential ribosome binding site at an appropriate distance and by a putative σ^{70} promoter sequence.

To determine whether the *torT* gene product plays a role in *tor* pathway expression, we disrupted the gene by an Ω interposon insertion by standard techniques (2, 23). First, the pLM1 plasmid DNA, which contains about 5 kb of *tor* region starting from the *Bgl*II site, was partially digested with *Bss*HII. The linearized plasmid was then blunted and ligated with a DNA fragment carrying the Ω interposon obtained from plasmid pHP45Ω cut with *Sma*I (21). One plasmid carrying the Ω interposon inserted within the 5′-proximal *torT* coding sequence was linearized and used to transform a *recB sbcB* strain as previously described (13). The *torT*::Ω chromosomal mutation from the resulting strain was subsequently transduced by P1 in strain MC4100 to yield strain LCB635 (Table 1). The *torT* gene disruption was confirmed by PCR experiments, performed with a primer located either at the beginning or at the end of the *torT* gene (T-Eco or T-Bam, see below) together with a primer complementary to both extremities of the Ω interposon (Ω primer, 5′-ATAAAGCTTGCTCAATCAAT CACCGG). The resulting LCB635 strain lost the inducible TMAO reductase activity. No protein cross-reacting with an antiserum raised against TorA, the TMAO reductase terminal enzyme (25), was detected under inducing conditions (data not shown). The *torT* mutation was then transduced into the *torA′-′lacZ* fusion-containing strain LCB620, to give rise to LCB637. A similar PCR-based strategy was used to ascertain the location of the Ω insertion. The expression of the fusion was then monitored by using LCB637 cells grown in minimal medium with or without 4 mM TMAO (Table 2). In this *torT*::Ω *torA′-′lacZ* strain, no β-galactosidase activity over the background level was observed whatever the growth conditions. Thus, a *torT* strain behaves identically to a *torR* strain (20, 28) in that

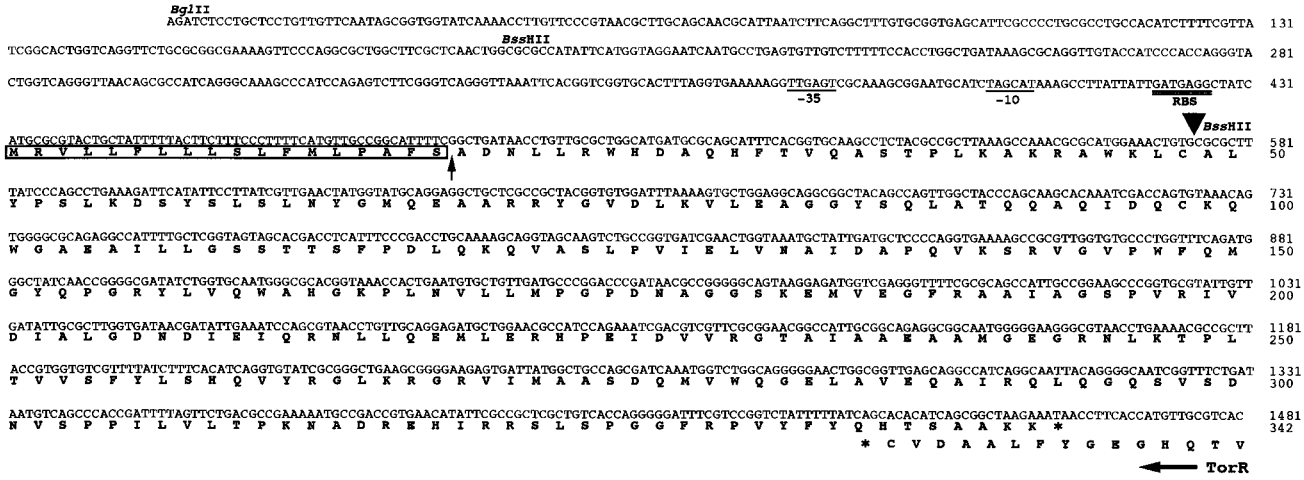

FIG. 1. Nucleotide and predicted amino acid sequences of the *torT* gene. The predicted amino acid sequence is given in a single-letter code below the DNA sequence. The amino acid sequence of the TorR C terminus is also indicated to highlight the *torR-torT* overlapping region. Asterisks mark stop codons; amino acids are aligned with the first nucleotide of each codon. The N-terminal signal sequence of TorT is boxed, and the signal-peptidase cleavage site is indicated by an arrow. *Bgl*II and *Bss*HII restriction sites are indicated. The numbers to the right of each row refer either to nucleotide positions relative to the *Bgl*II site or to the TorT amino acid positions. The putative −10 and −35 promoter regions are underlined, while the probable ribosome binding site is double underlined. The black triangle stands for the Ω insertion within the *torT* coding sequence of mutant strains.

they both lead to an absence of *tor* induction. These results provide evidence that the *torT* gene product, like the TorR protein, plays a regulatory role in *tor* operon expression.

To test the ability of the *torT*-containing pT1 plasmid to complement the Lac$^-$ phenotype of strain LCB637, the plasmid was introduced into this strain. Table 2 shows that the presence of *torT* in *trans* resulted in a normal induction of the *torA′-′lacZ* fusion. In addition, this result indicates that *torT* is likely to be expressed by a promoter located between the *Bgl*II site and the *torT* coding sequence.

As a last control and with the aim of overproducing TorT protein, the *torT* coding sequence was cloned downstream from the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *tac* promoter of plasmid pJF119EH (9). For this purpose, the *torT* sequence was first amplified by PCR from wild-type chromosomal DNA with an upstream oligonucleotide, T-Eco (5′-CGAATTCTTGAGGAGGCTATCATGCGCGTAC), composed of an *Eco*RI restriction site followed by a strong ribosome-binding site and by 19 nucleotides matching with the beginning of the *torT* coding sequence, and a downstream oligonucleotide, T-Bam (5′-ATGGATCCGGTTATTTCTTAGCCGCTGATG), containing a *Bam*HI cleavage site followed by 22 nucleotides complementary to the 3′ end of the *torT* coding sequence. After enzymatic hydrolysis, the *Eco*RI-*Bam*HI-amplified fragment was cloned at the polylinker cloning sites of pJF119EH to give plasmid pCJ6. The β-galactosidase activities of LCB637 cells carrying this plasmid were measured under various growth conditions (Table 2). Once again, the induction of the *torA′-′lacZ* fusion by TMAO was fully restored when a wild-type copy of *torT* was present. Since the growth of the cells was impaired by the addition of IPTG, these experiments were carried out in the absence of IPTG.

Taken together, these results strongly suggest that *tor* operon expression is strictly dependent on the function of the *torT* gene product.

Bypass of *torT* mutation by TorR overproduction. The fact that both TorR and TorT act as positive control elements for *tor* operon expression raised the question of a possible involvement of the two proteins in the same regulatory pathway. If one of the gene products is needed to activate the other, the absence of the activator would preclude *torCAD* induction. Yet, we sought to determine if the overproduction of the target protein would bypass the need for the activator, provided that

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
MC4100	*araD139* Δ(*lacIPOZYA-argF*)*U169 rpsL thi*	M. J. Casabadan
LCB620	MC4100 *torA8*::MudII 1734	13
LCB635	MC4100 *torT*::Ω	This work
LCB637	LCB620 *torT*::Ω	This work
620-621	LCB620 *torR49*::miniTn*10*	20
Plasmids		
pLM1	Bluescript KS$^+$ derivative carrying 5-kb *Bgl*II-*Bam*HI insert of the *tor* locus from pMCP2	13
pT1	Bluescript KS$^+$ derivative carrying 1.9-kb *Bgl*II-*Bst*EII insert obtained by deletion of the *Bst*EII-*Kpn*I region of pML4	M. Lepelletier
pCJ6	*torT* sequence inserted into pJF119EH	This work
pGS1	*torR* sequence inserted into pJF119EH	28

TABLE 2. Expression of the *torA'-'lacZ* chromosomal fusion in various strains

Strain[a]	β-Galactosidase activity (Miller units)[b] under condition			
	Anaerobiosis		Aerobiosis	
	+TMAO	−TMAO	+TMAO	−TMAO
LCB637	<5	<5	<5	<5
LCB637/pT1	291	<5	41	<5
LCB637/pCJ6	319	<5	61	<5
LCB637/pGS1	260	137	14	15
620-621/pCJ6	<5	<5	<5	<5

[a] Cells were grown aerobically or anaerobically on minimal medium (4) supplemented with glucose (0.2%) in the presence (+) or absence (−) of 4 mM TMAO. Ampicillin (50 $\mu g \cdot ml^{-1}$) was added to the medium for plasmid-carrying strains.

[b] β-Galactosidase activity was measured on whole cells as described by Miller (14). The data are the averages of at least three experiments.

the unactivated protein is partly functional in the unactivated state or that it can be activated by some other mechanism.

When the recombinant plasmid (pGS1) overexpressing *torR* was introduced into the *torT*::Ω *torA'-'lacZ* LCB637 strain, the overexpression of TorR led to *torA'-'lacZ* expression in the absence of TMAO but left the anaerobic control unchanged (Table 2). Thus, overproduction of TorR renders TorT nonessential for *tor* gene expression. That a high level of TorR protein can bypass the requirement for TorT is a genetic indication that the information normally flows from TorT to TorR rather than in the other direction. This was confirmed by the inability to express the fusion in a *torR torA'-'lacZ* strain (620-621) carrying the *torT* overexpressing plasmid pCJ6 whatever the induction conditions (Table 2). In addition to the *torR* epistatic effect, these results indicate that TorT is probably not involved in the anaerobic control since this control is still present in a *torT* strain overproducing TorR.

Subcellular location of TorT protein. The TorT protein is likely to be a periplasmic protein since the characteristic features of a prokaryotic signal peptide are present at its N terminus (22). The arginine basic residue after the initial methionine is followed by 12 uncharged, predominantly hydrophobic residues. Downstream from this hydrophobic core, a small region (P-A-F-S) could correspond to a signal peptidase cleavage site (Fig. 1).

To determine the subcellular location of TorT, we grew pCJ6-containing cells at 37°C in L broth. When the culture reached an A_{600} of 0.7, 1 mM IPTG was added for 2 h. Cells were then fractionated as previously described (17, 26). Figure 2 shows that a single band of proteins of about 35 kDa was found in the periplasmic fraction of bacteria carrying the *torT*-overexpressing plasmid pCJ6. This specific band was apparent neither in the periplasmic fraction of cells that do not overproduce TorT nor in the cytoplasm and membrane of bacteria able to overproduce TorT. Since the size of the specific band protein is in good agreement with the predicted molecular mass of the mature TorT protein (35.7 kDa), it appears that the TorT protein is located mainly in the periplasmic space.

To check whether the putative signal peptide was removed from the periplasmic protein, we determined the N-terminal amino acid sequence of this overproduced protein after transferring the gel containing the corresponding specific band onto a polyvinylidene difluoride membrane. The sequence obtained (ADNLLR) matched that of TorT from amino acid 19 to 24. This result clearly indicates that the TorT protein has a periplasmic location and that its signal peptide of 18 residues was removed during translocation.

Role played by TorT protein? Our data strongly suggest that, like TorR, TorT is a positive regulator involved in the induction of *tor* operon expression and that the two proteins are members of the same regulatory cascade. As pointed out by the genetic experiments described above, TorT certainly acts upstream from TorR, a response regulator able to directly bind the *tor* promoter (27). Since these proteins are located on opposite sides of the inner membrane, a transmembrane component is required for the putative signal to be transmitted from TorT to TorR. The still undescribed sensor protein, TorS, would be a perfect candidate for this role if one assumes that, like most sensor proteins, it is composed of a transmembrane N-terminal domain capable of interaction with external signals and a conserved cytoplasmic domain containing a specific His residue phosphorylated upon external stimulation (5, 18, 29). Accordingly, our working model proposes that, as TorT interacts with the periplasmic region of TorS, the autokinase activity of the TorS cytoplasmic domain rises and that this is followed by a transphosphorylation of the TorR regulator, which in turn triggers *tor* expression.

A gene regulatory pathway implicating a periplasmic protein in addition to the two regulatory components was found in a restricted number of cases such as virulence induction in *Agrobacterium tumefaciens* (7, 10, 30, 32). Indeed, *Agrobacterium* pathogenesis requires expression of the *vir* regulon, which is transcriptionally regulated by the VirA transmembrane protein kinase, the VirG response regulator, and the periplasmic sugar-binding protein, ChvE. Induction of the *vir* gene requires that certain plant phenolic compounds and monosaccharides be sensed through the VirA-VirG two-component regulatory system, whereas ChvE is required only for the induction by sugars. Mutants of *chvE* show an attenuated virulence and no sugar enhancement (6). This phenotype led Cangelosi and coauthors to speculate that the ChvE-sugar complex interacts directly with the periplasmic domain of VirA, resulting in increased *vir* induction in the presence of phenolic inducers. Additional genetic studies have provided support for this proposal (24). The ChvE amino acid sequence is homologous to that of the *E. coli* periplasmic galactose-binding protein (GBP) and, to a lesser extent, to that of the *E. coli* ribose-binding protein (RBP) (10, 24, 30). Thus, the ChvE protein is homologous to a family of periplasmic sugar-binding proteins involved in both chemotaxis and active transport. Interestingly,

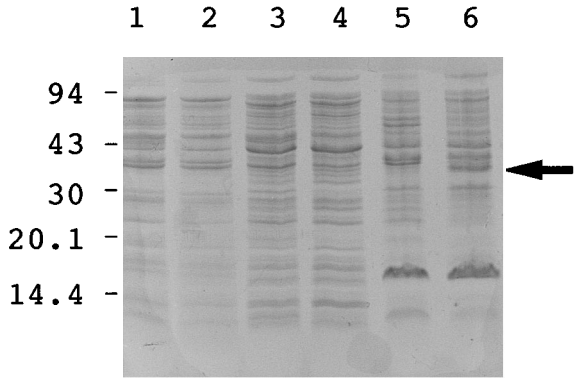

FIG. 2. Location of the TorT protein. Coomassie blue analysis of membranous (lanes 1 and 2), cytoplasmic (lanes 3 and 4), and periplasmic (lanes 5 and 6) *E. coli* extracts from cells carrying plasmid pCJ6 (lanes 2, 4, and 6), plasmid pJF119EH (lane 5), or no plasmid (lanes 1 and 3) after sodium dodecyl sulfate–20% polyacrylamide gel electrophoresis. The sizes of the molecular mass markers (not shown) are presented in kilodaltons at the left of the gel. The overproduced TorT band is indicated by an arrow.

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RBP    TLNNPFFVSLKDGAQKEADKLGYNLVVLDS  64
       :| ::: :||  | |  | : | :| ||::
TorT   SLKDSYSLSLNYGMQEAARRYGVDLKVLEA  82


RBP    ARERGEGFQQAVAAHKFNVLASQPADFDRIKGLNVMQNLL  200
       ::|  |||  |:|:   ::    :| |     |::|::|
TorT   SKEMVEGFRAAIAGSPVRIVDIALGDNDIEIQRNLLQEML  220

RBP    TAHPDVQAV--FAQNDEMALGALRALQTA  227
         ||::: |   |   | |:|  | | |:
TorT   ERHPEIDVVRGTAIAAEAAMGEGRNLKTP  249
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FIG. 3. Comparison of the amino acid sequences of *E. coli* periplasmic RBP and TorT protein. Vertical lines and colons represent identical and similar amino acid residues, respectively (similar residues are A, G, P, S, and T; E, D, Q, and N; F and Y; I, L, M, and V; and K and R). Dashes indicate gaps introduced to optimize the alignment initially obtained with the BLAST program (1). Numbers to the right of the sequences refer to amino acid residues.

the TorT protein, which exhibits no similarity with ChvE and GBP, shares significant homology with RBP within two restricted regions (Fig. 3; position 53 to 82, 33% identity; position 181 to 249, 30% identity). The similarity with RBP concerns about one third of the proteins; the remaining two thirds are completely divergent. The meaning of such a restricted homology is uncertain, but RBP and TorT may be, at least locally, structurally related (15). This homology, together with the fact that the four proteins mentioned above are members of transduction signal pathways and display a periplasmic location as well as a similar size, suggests that TorT might be a ligand-binding protein. If so, the initial process leading to *tor* operon expression would then be similar to that of bacterial chemotaxis or virulence as described above: an inducer first binds the periplasmic TorT protein, and then the protein-ligand complex interacts with the periplasmic domain of the putative TorS transmembrane protein to stimulate *tor* induction. Since TorR has been identified as the response regulator that mediates TMAO induction (28), TorT might be involved in TMAO signal transduction through TMAO sensing and binding. However, in a *torT* strain overproducing TorR, the addition of TMAO led to a twofold increase in β-galactosidase activity in anaerobiosis (Table 2). This result supports the idea that, in the absence of a functional *torT* gene, TMAO still behaves as an inducer. Moreover, *tor* expression is significantly lower in a *torT* strain than in the parental strain when both overproduce TorR (28). This is reminiscent of *Agrobacterium vir* induction, which requires two different inducers (certain monosaccharides and phenolic compounds) for maximal induction by the VirA sensor, the sugar signal being mediated through the periplasmic ChvE protein (6, 7, 32). In this line of thought, an attractive hypothesis is that the putative TorS sensor is contacted independently by TMAO and by the TorT protein bound to an unidentified inducer. Given the local homology between TorT and RBP, this hypothetical inducer could be a ribose-related sugar. Unfortunately, this hypothesis cannot account for the fact that the *tor* operon was expressed to normal levels in a medium devoid of any sugar as a carbon source (data not shown). Therefore, the current challenge is to identify the environmental signal which may be transduced through the TorT periplasmic protein to the *tor* regulatory network.

We are grateful to M. C. Pascal for valuable discussions and to F. Barras for helpful advice. We also thank M. Lepelletier for excellent technical assistance.

This work was supported by grants from the Centre National de la Recherche Scientifique and an MRT fellowship to C.J.

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