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# Transcriptional Analysis of the *tutE tutFDGH* Gene Cluster from *Thauera aromatica* Strain T1

PETER W. COSCHIGANO*

*Department of Biomedical Sciences and Program in Molecular and Cellular Biology, Ohio University, Athens, Ohio 45701-2979*

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**The denitrifying strain T1, identified as *Thauera aromatica*, is able to grow with toluene serving as its sole carbon source. Previous work identified two genes, *tutD* and *tutE*, that are involved in toluene metabolism. Two small open reading frames, *tutF* and *tutG*, which may also play a role in toluene metabolism, were also identified. The present work examines the transcriptional organization and regulation of these toluene utilization genes. Northern analysis indicates that the four genes are organized into two operons, *tutE* and *tutFDG*, and that both operons are regulated in response to toluene. Primer extension analysis has identified major transcriptional start sites located 177 bp upstream of the *tutE* translational start and 76 bp upstream of the *tutF* translational start. Furthermore, a fifth gene, *tutH*, has been identified immediately downstream of *tutG*. It is transcribed from the same start site as *tutFDG* and is predicted to code for a 286-amino-acid protein with a calculated molecular mass of about 31,800 Da. The TutH protein is predicted to have an ATP/GTP binding domain and is similar to the NorQ/NirQ family of proteins.**

Toluene is a hazardous substance that poses health risks to humans. A number of microorganisms that are able to metabolize this aromatic hydrocarbon under denitrifying conditions have been isolated and include *Thauera aromatica* K172 (27), *Azoarcus* sp. strain T (11, 17), and *T. aromatica* T1 (formerly known as strain T1) (12, 28). Biochemical studies with cell extracts of *T. aromatica* K172 and *Azoarcus* sp. strain T have shown that the first step in anaerobic toluene metabolism in these two organisms is the enzymatic formation of benzylsuccinate from toluene and fumarate (4, 6). This is a highly stereospecific reaction carried out by benzylsuccinate synthase, an enzyme recently isolated from *T. aromatica* K172 (4–6, 19). This purified enzyme has been shown to be an $\alpha_2\beta_2\gamma_2$ complex consisting of two subunits each of the BssA, BssB, and BssC proteins (19).

The genes coding for the benzylsuccinate synthase protein subunits have been cloned from *T. aromatica* K172 and designated *bssCAB* (19). Highly similar genes cloned from *T. aromatica* T1 and designated *tutFDG* (Fig. 1) most likely also code for a benzylsuccinate synthase (8). Based on similarities of the BssA and TutD proteins with pyruvate formate-lyase, and based on the reported mechanism for pyruvate formate-lyase (23, 24, 29), it has been proposed that these enzymes function by formation of a glycine free radical (8, 19). Biochemical work with the benzylsuccinate synthase enzyme from *T. aromatica* K172 and mutagenesis studies of the *tutD* gene of *T. aromatica* T1 support this mechanism of action (8, 19).

In addition to the *bssA* and *tutD* genes encoding benzylsuccinate synthase and its likely homologue, genes coding for a proposed benzylsuccinate synthase-activating enzyme have also been cloned from *T. aromatica* K172 and *T. aromatica* T1 and designated *bssD* and *tutE*, respectively (8, 19). The proposed function is based on the similarities of these gene products with pyruvate formate-lyase-activating enzymes (8, 19). The *bssD* and *tutE* genes are located upstream of and transcribed in the same direction as *bssA* and *tutD* (8, 19) (Fig. 1). It has been shown that in *T. aromatica* K172, the *bss* genes are grouped into an operon and transcribed as a single unit (19). Additionally, genes involved in the regulation of toluene metabolism in *T. aromatica* K172 and *T. aromatica* T1 have been identified (9, 18).

This report focuses on the transcriptional analysis of the *tutEFDG* gene cluster of *T. aromatica* T1. Since the transcription of the *bssDCAB* genes of *T. aromatica* K172 is induced by toluene (19) and anaerobic toluene metabolism is inducible in *T. aromatica* T1 (15), RNA analysis of the *tutEFDG* gene cluster of *T. aromatica* T1 was undertaken to determine if their regulation also occurs at the level of mRNA abundance. In addition, the transcriptional organization of these genes was examined and transcriptional start sites were identified by primer extension and nuclease protection assays. Finally, the identification and characterization of *tutH*, a new gene that codes for a putative ATP/GTP binding protein, are presented.

## MATERIALS AND METHODS

**Strains and DNA manipulation.** Isolation, characterization, and identification of *T. aromatica* T1, a gram-negative peritrichous denitrifying organism, have been reported previously (12, 28). The *Escherichia coli* strain XL-1 Blue (Stratagene, La Jolla, Calif.) was used to propagate DNA. The isolation and characterization of cosmid 13-6-4 were described previously (8, 9). To isolate DNA for sequencing, large-scale preparations were performed using the Qiagen (Santa Clarita, Calif.) maxiprep according to the manufacturer's instructions. DNA manipulations were carried out as described by Ausubel et al. (2).

**RNA preparation.** Wild-type *T. aromatica* T1 cells were grown under denitrifying conditions on a mineral salts medium (13) (vitamins and yeast extract omitted) with either pyruvate or toluene serving as the carbon source. When the density of the culture reached about $4 \times 10^7$ cells/ml, 35 ml of the culture was processed using the RNeasy Mini kit from Qiagen according to the manufacturer's instructions. Samples were run on a gel to confirm that there was no RNA degradation.

**Northern gel analysis.** About 0.25 μg of total RNA was run on a 0.8% agarose gel containing 1% formaldehyde (2). Ethidium bromide was added to each RNA sample to a final concentration of 31 μg/ml before denaturation and loading to allow visualization of the RNA without affecting the efficiency of RNA transfer to the membrane (22). After electrophoresis, the gels were treated with 0.05 N NaOH for 30 min, 0.1 M Tris (pH 7.5) for 30 min, and 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 30 min. RNA was transferred to a Hybond-N Membrane (Boehringer Mannheim, Indianapolis, Ind.) by capillary blotting overnight. The RNA was cross-linked to the membrane by baking at 80°C for 2 h in a vacuum oven. Antisense, digoxigenin-labeled, gene-specific

* Mailing address: Department of Biomedical Sciences, College of Osteopathic Medicine, Ohio University, Athens, OH 45701-2979. Phone: (740) 593-9488. Fax: (740) 597-2778. E-mail: Coschiga@ohiou.edu.

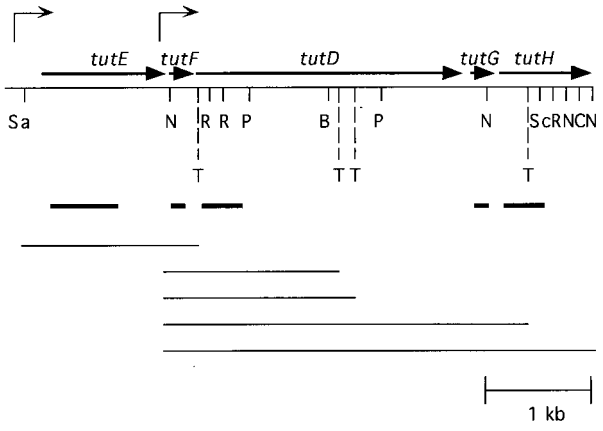


FIG. 1. Restriction map of the region of cosmid clone 13-6-4 that contains the *tutE tutFDGH* genes. The five identified open reading frames are indicated with thick arrows. Thin arrows above the map indicate the major transcriptional start sites identified. T, potential terminator site. The probes used for Northern analysis are also indicated below the map with thick lines. Potential transcripts (from the starts to the terminators) are shown below the probes with thin lines. Restriction site abbreviations: B, *Bam*HI; C, *Cla*I; N, *Nco*I; P, *Pst*I; R, *Eco*RI; Sa, *Sac*II; Sc, *Sac*I. Sites blocked by methylation are omitted.

DNA probes spanning nucleotides 97 to 398 of the predicted *tutD* coding region (302 nucleotides), 106 to 792 of the predicted *tutE* coding region (687 nucleotides), 14 to 152 of the predicted *tutF* coding region (139 nucleotides), 36 to 241 of the predicted *tutG* coding region (206 nucleotides), and 59 to 470 of the predicted *tutH* coding region (412 nucleotides) were made by PCR (20) and are indicated in Fig. 1. Prehybridization was performed at 42°C for at least 1 h in DIG Easy Hyb solution (Boehringer Mannheim). The probe was heated to 95°C and then added to the prehybridization mix at a final concentration of about 50 ng/ml. Hybridization was continued overnight at 42°C. The blots were washed twice with 2× SSC–0.1% sodium dodecyl sulfate (5 min, room temperature) and twice with 0.5× SSC–0.1% sodium dodecyl sulfate (5 min, 65°C). The probes were visualized on BioMax ML film (Eastman Kodak, Rochester, N.Y.) using the DIG High Prime DNA Labeling and Detection Starter Kit II (Boehringer Mannheim) according to the manufacturer's instructions with the chemiluminescence substrate CSPD. Digoxigenin-labeled RNA (Boehringer Mannheim) was also loaded on the gel to serve as a size marker.

**Primer extension analysis.** The Primer Extension System-AMV Reverse Transcriptase kit was purchased from Promega (Madison, Wis.) and used according to the manufacturer's instructions. About 2.5 μg of total RNA was used for each reaction. Primers F-PE1 (5′ CTG CTT GCA TGT GGT GGT TC 3′), binding from 4 to 23 bp downstream of the translational start of *tutF*, and E-PE3 (5′ GAT CCA CCA CGA CCA TAG AAG 3′), binding 5 bp upstream to 15 bp downstream of the translational start of *tutE*, were labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.) and [γ-$^{32}$P]ATP (New England Nuclear, Boston, Mass.). The labeled primers were used for both the primer extension reaction and the sequencing ladder. The primer extension reaction products and the sequencing ladder were run on a standard 8 M urea–5% polyacrylamide sequencing gel.

**Nuclease protection assay.** The Multi-NPA RNA/DNA/Oligo probe protection assay kit was purchased from Ambion (Austin, Tex.), and the manufacturer's standard procedure was followed. About 5 μg of total RNA was used for each reaction. Antisense gene-specific DNA probes of 354 bases (for *tutE*) or 623 bases (for *tutF*) spanning both the predicted transcriptional and translational start sites were synthesized by PCR (20) and labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-$^{32}$P]ATP (New England Nuclear). About $3 \times 10^5$ cpm of the probe was added to the assay mix. After completion of the reaction, the products were run on an 8 M urea–5% polyacrylamide gel.

**DNA sequence analysis.** DNA was sequenced (both strands) by the dideoxy method of Sanger et al. (26) with [α-$^{35}$S]dATP (New England Nuclear) serving as the label. Sequenase enzyme (modified T7 polymerase) and reagents were obtained in a Sequenase kit from Amersham Life Science (Arlington Heights, Ill.). The Bluescript vector and some primers used for sequence analysis were obtained from Stratagene. Synthetic oligonucleotide primers for the sequencing reactions were purchased from Life Technologies (Grand Island, N.Y.).

**Computer analysis.** Searches for protein sequence similarity were carried out against the nonredundant GenBank protein database using the BLAST 2.0.2 program (1). The Motif program (21) was used to identify patterns in the protein sequences that could have a functional role. Potential factor-independent transcriptional terminator sites were identified with the Terminator program (7). Multiple sequence alignments were performed with the Lasergene software package from DNASTAR (Madison, Wis.).

**Nucleotide sequence accession number.** The nucleotide sequence reported here has been submitted to the GenBank database and assigned accession number AF113168.

## RESULTS AND DISCUSSION

**Transcriptional regulation of the toluene utilization genes.** Northern analysis was used to examine the regulation of the toluene utilization genes of *T. aromatica* T1. Intense bands were detected when *tutD*, *tutE*, *tutG* (formerly open reading frame [ORF] 4) (8), and *tutF* (formerly ORF 2) (8) gene-specific probes were hybridized to aliquots of RNA isolated from toluene-grown cells (Fig. 2, lanes T). In contrast, no bands were detected by any of the *tut* gene-specific probes when aliquots of RNA isolated from pyruvate-grown cells were used (Fig. 2, lanes P). Since equal amounts of total RNA were loaded in the T and P lanes (as confirmed by ethidium bromide visualization of the 16S and 23S rRNA bands before transfer [data not shown]), these results indicate that the *tut* genes are induced by toluene. The *bssDCAB* genes of *T. aromatica* K172, which are similar to *tutE tutFDG*, are also regulated in response to toluene (19).

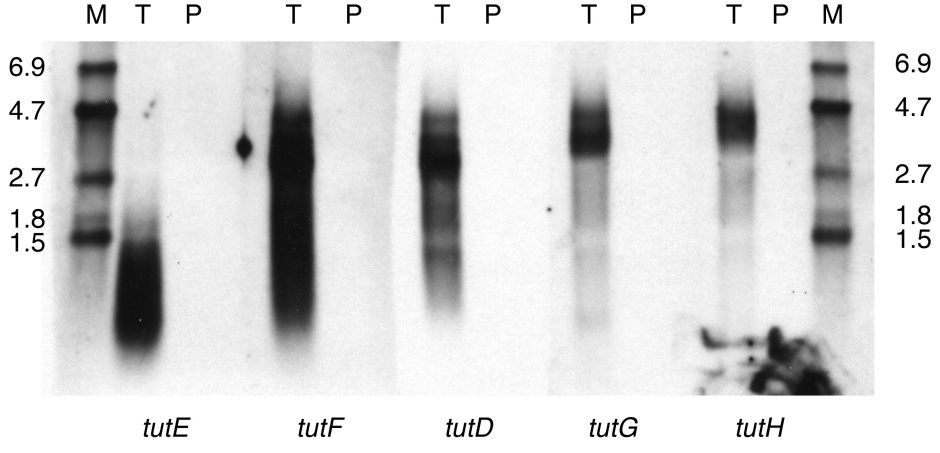


FIG. 2. Northern analysis of total RNA isolated from cells grown under denitrifying conditions with either toluene (T) or pyruvate (P) as the carbon source and visualized with gene-specific probes derived from *tutE*, *tutF*, *tutD*, *tutG*, or *tutH*. Samples of digoxigenin-labeled RNA were included to serve as size markers (M) and are labeled (in kilobases) on the sides.

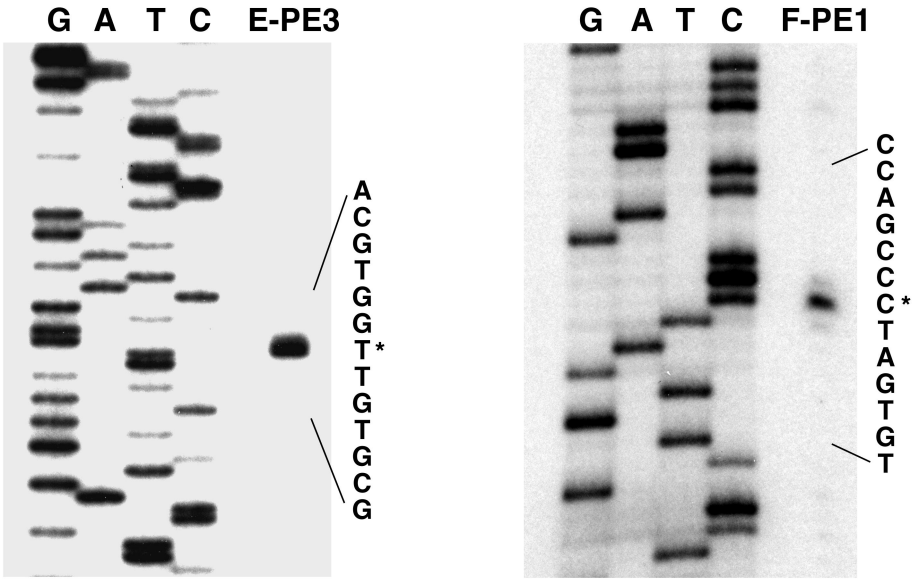


FIG. 3. Primer extension analysis to map the transcriptional start sites of the *tutE* and *tutF* genes. End-labeled primer E-PE3 was used to identify the *tutE* start of transcription and end-labeled primer F-PE1 was used to identify the *tutF* start of transcription. The same primers were used to generate the sequencing ladder by the dideoxy method (lanes marked G, A, T, and C). The sequence encompassing the major transcriptional start (marked with an asterisk) is enlarged.

It can also be seen from Fig. 2 that the banding pattern observed with the *tutE* gene-specific probe is distinct from the patterns observed with the *tutF*, *tutD*, and *tutG* gene-specific probes. The transcripts observed using the *tutE* probe are predominantly less than 1.5 kb in size, with a faint band observed at about 1.7 kb. Various sizes of mRNA transcripts are also observed with the other three probes, but the largest transcripts are approximately 4.5 kb in size. The banding patterns suggest that *tutF*, *tutD*, and *tutG* are part of one transcriptional unit and that *tutE* is a separate transcriptional unit.

**Identification of a new toluene utilization gene.** Since the maximum size of the *tutF*, *tutD*, and *tutG* mRNA transcripts (about 4.5 kb) was significantly larger than needed to code for these genes (about 3.1 kb), an examination of the DNA downstream of *tutG* was undertaken. An additional open reading frame was identified and designated *tutH*. Figure 2 includes the results of a Northern analysis in which a *tutH* gene-specific probe was used to identify transcripts from toluene-grown cells. The pattern observed with RNA isolated from toluene grown cells with the *tutH* probe was similar to that seen with the *tutF*, *tutD*, and *tutG* probes (Fig. 2). In addition, the *tutH* probe did not detect any transcripts in RNA isolated from pyruvate-grown cells, indicating that *tutH* is also induced by toluene (Fig. 2).

**Identification of the transcriptional start sites.** The Northern analysis described above suggested that the *tutF*, *tutD*, *tutG*, and *tutH* genes are likely to be contained within a single transcriptional unit, while the *tutE* gene is separate. Thus, primer extension and nuclease protection analyses were undertaken to identify the transcriptional start site(s) present for each gene.

Figure 3 shows the results of a primer extension reaction using RNA isolated from toluene-grown cells and the E-PE3 primer, in which the predicted *tutE* translational start is contained. The major transcriptional start site is located 177 bp upstream of the *tutE* translational start. This same start site was also observed when a nuclease protection assay was carried out with a DNA probe spanning the *tutE* translational start (data not shown). Minor start sites (all of comparable intensity) were also observed 178 bp upstream (Fig. 3) and in the region 119 to 124 bp upstream of the *tutE* translational start (data not shown). None of these sites were observed when the analyses were performed with RNA isolated from pyruvate-grown cells (data not shown).

As can also be seen in Fig. 3, results of a primer extension reaction using RNA isolated from toluene-grown cells and the F-PE1 primer (located just downstream of the predicted *tutF* translational start site) identified a major transcriptional start site 76 bp upstream of the *tutF* translational start. This site is located within the *tutE* coding region. This start site was also observed when a nuclease protection assay was carried out with a DNA probe spanning this region of the *tutF* translational start (data not shown). Minor transcriptional start sites (all of comparable intensity) were observed 75 and 77 bp upstream (Fig. 3) and in the region 125 to 129 bp upstream of the *tutF* translational start site (data not shown). None of these sites were observed when the analyses were performed with RNA isolated from pyruvate-grown cells (data not shown). These results are consistent with the results of the Northern analysis, indicating that the *tutE* transcript is separate from the *tutFDGH* transcript.

A primer extension reaction carried out with a primer located downstream of the predicted *tutD* translational start and a nuclease protection assay carried out with a DNA probe spanning the *tutD* translational start did not identify a transcriptional start immediately upstream of *tutD*. Instead, these reactions did identify the same start site located upstream of *tutF* (data not shown). Primer extension reactions carried out with primers located downstream of the predicted *tutG* and *tutH* translational starts and nuclease protection assays carried out with DNA probes spanning these translational start sites failed to identify transcriptional start sites immediately upstream of these genes (data not shown). The start site identified preceding *tutF* could not be verified for *tutG* and *tutH* due to its considerable distance from these genes (about 2.9 and 3.2 kb, respectively). However, the RNA analyses suggest that the *tutF*, *tutD*, *tutG*, and *tutH* genes are transcribed as a single unit from one start site. Thus, the variety of mRNA transcript sizes

seen in the Northern analyses is likely not due to different transcriptional start sites but may be due to different transcriptional termination sites.

It is not surprising that the *tutFDG* genes, as well as the similar *bssCAB* genes of *T. aromatica* K172, which encode subunits of the benzylsuccinate synthase enzyme and are included in the *bssDCAB* gene cluster (19), are located in single operons. However, since both the *tutE* and *bssD* gene products are predicted to function as activators that enzymatically form a glycine free radical in the proteins encoded by the *tutD* and *bssA* genes, respectively, it might be expected that the activator proteins would be needed in smaller amounts than the activated proteins. Thus, organization into separate transcriptional units might be useful for regulatory purposes. Indeed, in the case of the pyruvate formate-lyase systems of *E. coli*, *Haemophilus influenzae*, and *Clostridium pasteurianum*, which show sequence similarities to the *tutD* and *tutE* genes and the *bssA* and *bssD* genes, the pyruvate formate-lyase-activating protein is located on a different transcriptional unit from the pyruvate formate-lyase (14, 24, 30). It is not clear why the *tutE tutFDG* and the *bssDCAB* gene clusters, coding for homologous proteins which are expected to carry out the same chemical reaction in two strains of the same bacterial species, would be organized in such different manners.

**Computer analysis of terminator and promoter regions.** A search for potential factor-independent terminator-like sequences using the Terminator program (7) led to the identification of the four sites shown in Fig. 1. An additional terminator site is presumed to reside downstream of *tutH*. It is predicted that a transcript starting upstream of *tutE* and ending at the terminator past *tutF* would be about 1.5 kb and would be visualized by both the *tutE* and *tutF* gene-specific probes. Similarly, transcripts beginning at the start site upstream of *tutF* are predicted to be about 1.6, 1.8, 3.6, and greater than 4.3 kb, depending on which terminator is used. The *tutF* probe would be expected to identify all of these transcripts, while the *tutG* and *tutH* gene-specific probes would be expected to identify only the two largest. Indeed, results from the Northern analyses are consistent with the predicted locations of these putative terminators (Fig. 2). This computer analysis does not identify factor-dependent terminators that may also be present.

An examination of the regions immediately upstream of the defined transcriptional start sites failed to identify any consensus −35 or −10 sites. Additionally, a search failed to identify homology between the regions upstream of both *tutE* and *tutF* and any known bacterial promoter regions. A pairwise comparison of only the sequences upstream of *tutE* and *tutF* did identify a number of similar regions, ranging from 20 to 60 nucleotides in length. The significance (if any) of these sites is currently under investigation.

**Sequence analysis of *tutH*.** Since the 4,905-bp *Sac*II/*Eco*RI fragment of cosmid 13-6-4 (GenBank accession number AF036765) (8, 9) did not contain the complete sequence of the *tutH* gene, an additional 381 bp of this cosmid was sequenced on both strands. The 1,018-bp *Nco*I fragment (part of which is contained in the *Sac*II/*Eco*RI fragment previously reported) containing the *tutH* sequence has been deposited in GenBank. Analysis of this sequence identified the complete *tutH* coding region, whose predicted protein product is 286 amino acids. The TutH protein has a calculated molecular mass of about 31,800 Da and a predicted pI of 5.4.

The BLAST program (1) was used to identify proteins similar to the predicted TutH protein. The four proteins with the highest degree of similarity were NorQ from *Paracoccus halodenitrificans* (25), *Paracoccus denitrificans* (10), and *Rhodobacter sphaeroides* (3) and NirQ from *Pseudomonas stutzeri* (16). The BLAST program calculated that these proteins are 27, 28, 27, and 22% identical (over nearly their entire sequence) to TutH, respectively.

The TutH protein sequence was also subjected to a Motif analysis (21). Amino acids 47 to 54 were identified as a putative ATP/GTP binding domain, a region that is conserved in the NorQ and NirQ proteins. This observation suggests that the NorQ-NirQ family of proteins and the TutH protein may use a similar mechanism involving ATP/GTP binding. It is possible that these proteins function in similar manners in their different systems and that this ATP/GTP binding is necessary for their function. The roles of NorQ and NirQ have not been elucidated, but studies of the NirQ protein from *P. stutzeri* suggest that it may posttranslationally regulate the activity of nitric oxide reductase (16). Based on results obtained with *R. sphaeroides*, it has been suggested that the NorQ protein may be involved in the assembly of the active nitric oxide reductase enzyme complex (3). The *bssCAB* gene products of *T. aromatica* K172, which are similar to those of the *tutFDG* genes, have been shown to form a complex (19). Based on this comparison, it is expected that the *tutFDG* gene products of *T. aromatica* T1 also form a complex. Thus, it can be speculated that the role of TutH is to posttranslationally modify one or more of the TutF, TutD, and TutG proteins and/or aid in the assembly of an active enzyme complex containing these proteins. Interestingly, a gene showing similarity to the *tutH* gene has not been observed in *T. aromatica* K172 (19). What role, if any, the *tutH* gene product plays in anaerobic toluene metabolism remains to be determined.

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