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Identification and Sequence Analysis of Two Regulatory Genes Involved in Anaerobic Toluene Metabolism by Strain T1

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T1 is a denitrifying bacterium isolated for its ability to grow with toluene serving as the sole carbon source. Mutants of this strain that have defects in the toluene utilization pathway have been isolated and have been separated into classes based on growth phenotypes. A cosmid clone has been isolated by complementing the *tutB16* (for toluene utilization) mutation. The complementing gene has been localized to a 3.3-kb DNA fragment. An additional open reading frame upstream of the *tutB* gene has also been identified and is designated *tutC*. The nucleotide sequence and the predicted amino acid translation of the 6.4-kb DNA fragment that contains these genes are presented. The *tutB* and *tutC* gene products of strain T1 have homology to members of the two-component sensor-regulator family and are proposed to play a role in the regulation of toluene metabolic genes of strain T1. To our knowledge, this is the first published report of the isolation of mutants defective in anaerobic aromatic hydrocarbon degradation. Additionally, we report for the first time the cloning of genes involved in an anaerobic aromatic hydrocarbon degradation pathway.

Toluene, along with benzene and xylenes, is a common contaminant of groundwater and surface water. These compounds are the most water soluble of all gasoline components and can enter aquatic environments from many sources such as gasoline underground storage tanks, leaks, and spills. Toluene has been classified by the U.S. Environmental Protection Agency as a priority pollutant due to its ability to depress the central nervous system and to enhance the effect of known carcinogens (37).

A number of bacterial strains which can degrade toluene have been isolated. The toluene degradation pathway of some of the aerobic strains has been well studied, and work from a number of laboratories has identified five different pathways of aerobic toluene metabolism. Extensive biochemical, genetic, and molecular analyses of these aerobic pathways have resulted in detailed knowledge of enzyme mechanisms and genetic regulation (for a recent review, see reference 39).

Anaerobic toluene-degrading bacterial strains have also been isolated but have not been studied as extensively as the aerobic strains (for a review, see reference 14). Recently, several new isolates have been identified as denitrifiers and members of the genus *Azoarcus* (16, 38). Strain T1 has been isolated in our laboratory for its ability to degrade toluene under anaerobic, denitrifying conditions (11). The morphological, physiological, and genomic properties of strain T1 (including sequence analysis of 16S rRNA) suggest a relationship to the genus *Thauera* of the beta branch of *Proteobacteria* (24). A manuscript describing T1 taxonomic properties and the criteria for its classification is in preparation (30).

Studies with strain T1 have shown that it mineralizes toluene while using nitrate as the electron acceptor (11). It has also been shown that the toluene-utilizing pathway of this strain is inducible (15). A pathway for the utilization of toluene in strain T1 has been proposed and is presented in Fig. 1 (10). Toluene is believed to be metabolized via an acetyl coenzyme A (acetyl-CoA) attack on the methyl group to form phenylpropionyl-CoA (also known as hydrocinnamoyl-CoA), which is then converted via β-oxidation to form benzoyl-CoA. A side reaction of the proposed pathway is a succinyl-CoA attack on the methyl group of toluene, resulting in the production of the dead-end compounds benzylsuccinic acid and benzylfumaric acid. These dead-end compounds have previously been identified by gas chromatography-mass spectrometry (10).

In this study, we isolated mutant strains of T1 that were unable to utilize toluene as the sole source of carbon and energy. We report the cloning, nucleotide sequence, and predicted amino acid sequence of two genes, one that complements the *tutB16* mutation and a second gene (*tutC*) located immediately upstream of the first. Analysis of these amino acid sequences revealed similarities to members of two-component regulatory systems (29). Based on this homology, we suggest that the *tutB* and *tutC* gene products are involved in the regulation of the toluene utilization pathway of strain T1.

MATERIALS AND METHODS

Strains and plasmids. Isolation, characterization, and a description of strain T1, a gram-negative peritrichously flagellated denitrifying organism, have been presented previously (11). *Escherichia coli* HB101 (5), XL-1 Kan Blue (Stratagene, La Jolla, Calif.), and XL-1 Blue (Stratagene), used to propagate and transfer DNA, were transformed by the calcium chloride technique (25, 26) or were purchased from the company as competent cells. Strain HB101(pRK2013) (Kan^r) (13) contains a helper plasmid that permitted mobilization of cosmids and plasmids into the T1 strain background.

Plasmids used in this study include pLAFR3 (36) for construction of the genomic cosmid library, pRK415 (21) for construction of subclones and matings, and the pBluescript vector (Stratagene) for subcloning and preparation of DNA fragments.

Media. Strain T1 and all strains derived from T1 were grown on either brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) medium or a mineral salts medium (12) (vitamins and yeast extract omitted). Unless otherwise specified, toluene (0.3 to 0.5 mM) or pyruvic acid (5 mM) was used as the carbon source to supplement the minimal medium. Nitrate was supplied to a concentration of 10 to 20 mM unless otherwise specified. Plates always contained 2% Noble agar (Difco Laboratories). Liquid medium was prepared and placed in serum bottles, which were then tightly stoppered with Teflon-coated butyl rubber and aluminum crimp seals. Anaerobic conditions were generated by evacuation and subsequent filling of the bottles with argon. This process was performed a

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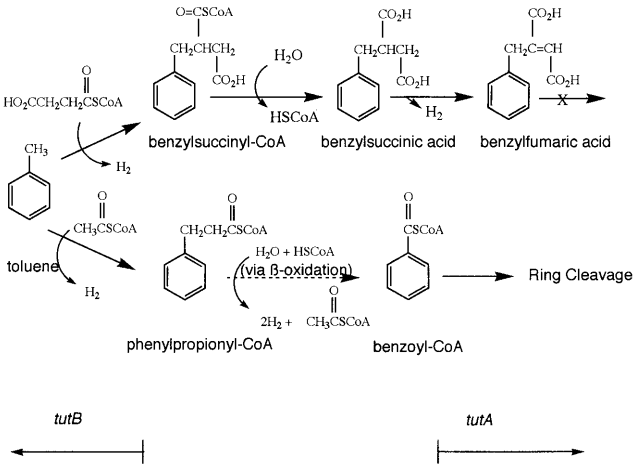

FIG. 1. Pathway of toluene utilization as proposed by Evans et al. (10). The upper branch of the pathway shows the conversion of toluene to the dead-end products benzylsuccinic acid and benzylfumaric acid. The lower branch of the pathway shows the mineralization of toluene via benzoyl-CoA. Proposed regions of the pathway affected in the *tutA* and *tutB* classes of mutants are also indicated.

total of four times. *E. coli* was grown in Luria-Bertani agar or broth (LB) (4) or on BHI agar plates.

The antibiotics kanamycin (used at 50 μg/ml) and tetracycline (used at 25 μg/ml) were supplied where indicated. A 12.5-mg/ml stock of tetracycline was made in ethanol. Upon addition to minimal media, the tetracycline served to select for the cosmid while the ethanol (final concentration of approximately 17 mM) served as the carbon source for the transconjugant strains.

Mutagenesis. Mutagenesis was carried out on strain T1 under aerobic conditions in a manner similar to that described by Miller (28) for *E. coli*. Strain T1 was grown in a rich medium (BHI plus nitrate), washed, and resuspended in 100 mM sodium citrate buffer (pH 5.5) to a cell density of about 3.5×10^8 cells/ml. The cell suspensions were treated with nitrosoguanidine (final concentration of 50 μg/ml), and aliquots were removed at various times. The mutagenized cells were harvested by centrifugation, washed with 100 mM potassium phosphate buffer (pH 7.0) to remove the nitrosoguanidine, and then resuspended in the phosphate buffer. The treated cells were subjected to titer determination on BHI plates to establish a killing curve. The treatment group that resulted in about 50% killing was used for the isolation of mutants. Treated cells were diluted in phosphate buffer to yield 100 to 200 colonies per plate and spread onto minimal medium plates supplemented with nitrate and pyruvic acid. After 5 days of incubation (30°C anoxic), colonies were replica plated to rich medium and minimal medium plus nitrate, with toluene supplied in the vapor phase (11). The plates were placed in an anaerobic incubation jar, which was then sealed and filled with hydrogen gas (to 12 lb/in^2). In the presence of a palladium catalyst, oxygen is removed by reaction with the hydrogen, producing water and resulting in an anoxic atmosphere. After 5 days of anaerobic incubation (30°C), colonies that grew on the rich medium but not on the minimal medium with nitrate and toluene were picked and streaked onto rich plates. The strains were retested for the ability to grow with toluene serving as the sole carbon source in both liquid and solid media. The strains were later tested for the ability to utilize toluene and produce the dead-end products benzylfumaric acid and benzylsuccinic acid in liquid culture.

Chemicals. Tetracycline was purchased from Fluka (Ronkonkoma, N.Y.). Kanamycin and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (nitrosoguanidine) were obtained from Sigma (St. Louis, Mo.).

Construction of the cosmid library. Strain T1 was grown under anaerobic conditions in 500 ml of minimal medium containing nitrate and ethanol, and genomic DNA was isolated by the method of Staskawicz et al. (35). The DNA was purified by two successive CsCl gradient centrifugations. A partial digest of the DNA with *Sau*3AI was carried out, and fragments of 15 to 25 kb were isolated on a 10 to 40% glycerol gradient. These fragments were ligated into the *Bam*HI site of pLAFR3. The resulting ligation mix was packaged into phage heads by using a Packagene kit (Promega, Madison, Wis.). *E. coli* HB101 was infected with the phage and plated onto LB-tetracycline plates. The resulting 750 colonies were streaked on plates containing the same medium, and the isolates served as the genomic library for obtaining the cosmid clone.

Triparental matings. Triparental matings were carried out essentially as described previously (7). Mutants of strain T1 were grown for 3 days in minimal medium containing nitrate and pyruvic acid. HB101 (or XL-1 Kan Blue) carrying the donor cosmid or plasmid was grown in LB-tetracycline overnight. HB101(pRK2013) was grown in LB-kanamycin overnight. A 1-ml sample of each culture was centrifuged and resuspended in an equal volume of 100 mM phosphate buffer (pH 7). Then 10 μl of each culture was spotted (one on top of the other) onto a BHI-nitrate plate. After a 3-day incubation at 30°C in an anoxic environment, the resulting growth was scraped off the plate, resuspended in phosphate buffer, and spotted onto a minimal agar plate containing pyruvic acid, nitrate, ethanol, and tetracycline to select for transconjugants. After another 3-day incubation, cells from the resultant growth were streaked onto the same medium and grown in a sealed jar in the absence of oxygen. After 3 days of incubation, single transconjugant colonies were isolated from these plates and tested for complementation.

Restriction mapping and subcloning. DNA manipulations were carried out as described by Maniatis et al. (26). All enzymes were obtained from New England BioLabs (Beverely, Mass.). Cosmid 13-6-4 was the original clone isolated. Plasmid pPWC1-HSma was constructed in two steps. The first step entailed deleting the *Hin*dIII fragment of 13-6-4 (from the *Hin*dIII site internal to the insert to the *Hin*dIII site [not shown in Fig. 2] in the pLAFR vector just beyond [to the right of] the *Bam*HI site) by digestion of 13-6-4 with *Hin*dIII and subsequent religation. The resulting cosmid (13-6-4-ΔH) was digested with *Hin*dIII and *Sma*I, and the 3.8-kb DNA fragment was isolated and inserted into *Hin*dIII-*Sma*I-digested pBluescript. The *Hin*dIII-*Sma*I fragment was transferred to pRK415 by cutting both plasmids with the enzymes *Xba*I and *Kpn*I and then isolating and ligating the fragments. The resulting plasmid was designated pPWC2-HSma (see Fig. 2). Plasmid pPWC1-C_s was constructed by cutting 13-6-4 with *Cla*I, isolating the small (3.3-kb) DNA fragment, and inserting it into *Cla*I-digested, calf intestinal alkaline phosphatase-treated pBluescript. The *Cla*I fragment was transferred into pRK415 by cutting pPWC1-C_s and the vector with *Xba*I and *Kpn*I (to generate pPWC2-C_s) or with *Kpn*I and *Eco*RI (to generate pPWC2-C_s′, the reverse orientation of pPWC2-C_s) and ligating.

Restriction mapping was carried out with fragments inserted into the pBluescript vector to facilitate identification of restriction sites and to help place the sites on a restriction map. Digests were run on different percentages of agarose gels with size standards to estimate the size of the fragments and to locate restriction sites.

Testing for complementation. Cosmid clones and subclones constructed in pLAFR3 or plasmid subclones constructed in pRK415 were mated into the *tutB16* mutant background via the triparental-mating technique. The resultant transconjugant strain was tested to determine if the subclone complements the mutation. First, the transconjugants were streaked onto minimal-plus-nitrate plates in which toluene was supplied in the vapor phase (11). After 5 to 7 days of anaerobic incubation (30°C), the subclones were scored for the ability to restore growth on toluene to the mutants. The transconjugants were also grown in sealed 50-ml serum bottles of minimal liquid medium plus nitrate (10 mM), pyruvic acid (1 mM), and toluene (0.4 mM) with an argon headspace. After 3 to 4 days of incubation (30°C), samples were withdrawn for toluene and dead-end product analysis (see below). The clones were scored for the ability to restore toluene utilization (in the presence of pyruvate) in liquid culture and for the ability to restore production of the dead-end metabolites under the same conditions to the mutants. If the transconjugant was positive for all three of these tests, the subclone was considered to complement the mutation.

Toluene analysis. Samples (1 ml) of the culture to be tested were withdrawn anaerobically and added to 400 μl of pentane containing 1 mM fluorobenzene as an internal standard in a sample vial. Then 1 μl of the organic phase (into which toluene had been extracted) was injected with a CTC A200S autosampler (LEAP Technologies, Chapel Hill, N.C.) into an HP5890 gas chromatograph (Hewlett-Packard, Palo Alto, Calif.) equipped with a flame ionization detector, a DB-WAX column (J&W Scientific, Folsom, Calif.) and helium as the carrier gas. The injector temperature was set at 250°C, the detector temperature was set at 300°C, and the column temperature was set at 35°C. The amount of toluene present in each sample was quantified by comparison to external standards with the Chemstation software (Hewlett-Packard).

Analysis of dead-end products. Samples of the culture were withdrawn anaerobically with a sterile syringe flushed with argon. The samples were centrifuged (5 min in a microcentrifuge), and the supernatant was filtered through a 0.45-μm-pore-size filter (Millipore Corp., Bedford, Mass.) into a sample vial. Samples were analyzed by high-pressure liquid chromatography with a System Gold high-pressure liquid chromatograph (Beckman, Fullerton, Calif.) equipped with a Gilson (Middleton, Wis.) autosampler and a C_{18} column (250 by 4.6 mm; particle size, 5 μm [Beckman]) with UV detection at 260 nm. The mobile phase was 30:68:2 methanol-water-acetic acid (30:68:2, vol/vol/vol) (15) at a flow rate of 1 ml/min. Peaks were identified by comparison to the external standards benzylmaleic acid and benzylsuccinic acid (10).

Plasmid DNA preparation. In general, DNA plasmid minipreparations were performed by the boiling method of Holmes and Quigley (19). When larger-scale preparations were needed, Qiagen (Chatsworth, Calif.) maxipreparations were carried out as specified by the manufacturer.

DNA sequence analysis. DNA was sequenced (both strands) by the dideoxy method of Sanger et al. (32), with [α-^{35}S]dATP serving as the label. Sequenase enzyme (modified T7 polymerase) and reagents were obtained in a Sequenase kit from U.S. Biochemicals (Cleveland, Ohio). The Bluescript vector and the T3, T7, −20, and M13 reverse primers used for sequence analysis were obtained from Stratagene. An Erase-a-Base System (Promega) was used to generate deletions of the cloned DNA inserted in the Bluescript vector for sequence analysis. Synthetic oligonucleotide primers were also purchased so that sequence data

TABLE 1. Characterization of *tut* mutants by growth phenotype and dead-end product accumulation

Class	Mutant allele	Growth[a] on:			Dead-end product accumulation[b]
		Toluene	Phenylpropionic acid	Benzoic acid	
Wild type		+	+	+	+
tutA	*11*	−	−	−	+
tutA	*18*	−	−	−	+
tutA	*20*	−	−	−	±
tutB	*16*	−	+	+	±
tutB	*17*	−	+	+	−
tutB	*19*	−	+	+	ND[c]
tutB	*21*	−	+	+	−

[a] +, growth in 3 to 5 days; −, no growth.
[b] +, production of the dead-end compounds benzylfumaric acid and benzylsuccinic acid to the same extent as in the wild type; −, no production of the dead-end compounds; ±, production of dead-end compounds to about one-third the level in the wild-type strain.
[c] ND, not determined.

could be obtained to fill in gaps not covered by the deletions. Searches for protein sequence similarity were carried out with the Swissprot database (release 32.0) of protein sequences by using the FASTA program (31) in software package version 7.2 (GCG Software, Madison, Wis.). Multiple-sequence alignment was 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 U57900.

RESULTS

Isolation of *tut* mutants. Cells of strain T1 were grown and mutagenized with nitrosoguanidine as described in Materials and Methods. Mutants were isolated from the treatment group that resulted in about 50% killing. Cells were diluted and plated onto minimal medium supplemented with nitrate and pyruvic acid to a density of about 100 to 200 colonies per plate. After about 5 days of incubation at 30°C in the absence of oxygen, the colonies were replica plated to both rich medium and minimal medium with nitrate and with toluene supplied in the vapor phase. After incubation, colonies that grew on the rich medium but failed to grown on the minimal medium with nitrate and toluene were chosen for further study. Of about 10,000 colonies screened, 32 candidates were isolated in this manner. These 32 mutant candidates were again tested for their ability to grow on minimal medium supplemented with nitrate and toluene both in liquid and on plates. Retesting the candidates identified seven mutants which were truly defective for toluene utilization. These seven were designated *tut* mutants for their defect in toluene utilization.

Characterization of *tut* mutants. The seven *tut* mutants were tested for their ability to grow on various carbon sources. Table 1 shows that four of the mutants are able to use benzoic acid and phenylpropionic acid as the sole carbon source while three are not able to use either substrate. Based on this observation, the first group is predicted to be blocked early in the toluene utilization pathway and is designated *tutB* (Fig. 1). The second group is blocked later in the pathway, probably in benzoic acid utilization, and is designated *tutA* (Fig. 1). These designations are not meant to imply that all mutants in a particular group are defective in the same gene or in the same step of the pathway, only that they utilize the same range of substrates.

The *tut* mutants were also tested for their ability to metabolize toluene when provided with both toluene and pyruvic acid in liquid media. Pyruvic acid was added to ensure that the transconjugants grew and that there was no selective pressure for reversion of the mutation to occur. Although the *tutB16* mutant metabolized toluene slightly, none of the *tutB* mutants tested were able to metabolize toluene to the same extent as the wild-type control did. Similarly, the *tutB* mutants did not produce significant amounts of the dead-end products benzylsuccinic acid and benzylfumaric acid (Table 1). Members of the *tutA* class of mutants were able to both metabolize toluene and produce the dead-end products. This result indicates that the *tutB* mutants are blocked in a step(s) that is common to both the metabolic degradation of toluene and the side reaction that produces the dead-end compounds or in the regulation of such a step(s).

Generation of the T1 DNA library. It has previously been shown that pLAFR3-derived cosmids can be transferred into and stably maintained in the strain T1 background (7). Consequently, this vector was chosen for the construction of a genomic DNA library of strain T1. Genomic DNA was isolated from strain T1 as described in Materials and Methods. A partial digest of the genomic DNA was carried out with *Sau*3AI, and fragments of between 15 and 25 kb were isolated. These fragments were ligated into the *Bam*HI site of pLAFR3. The resulting ligation mix was packaged into lambda phage heads and used to infect *E. coli* HB101. About 750 tetracycline-resistant *E. coli* colonies were picked and formed the genomic library used to isolate clones that complement the *tut* mutations.

Isolation of a clone that complements *tutB16*. The genomic T1 library constructed in pLAFR3 was introduced via a triparental mating into a T1-derived strain carrying the *tutB16* mutation (see Materials and Methods). The donors for all the cosmids were *E. coli* HB101-derived strains, while *E. coli* HB101 carrying plasmid pRK2013 served as the helper to mobilize the cosmids. Transconjugants were selected on minimal medium supplemented with nitrate, pyruvic acid, and tetracycline and then screened for the ability to grow with toluene serving as the sole carbon source. One cosmid, designated 13-6-4, restored the ability of the *tutB16*-carrying T1 strain to grow on toluene. This cosmid also restored the ability of the mutant strain to metabolize toluene in the presence of pyruvic acid in liquid culture and to produce the dead-end products benzylsuccinic acid and benzylfumaric acid in this culture. This cosmid was used for further subcloning and restriction mapping to specifically identify the region containing the complementing gene.

Subcloning and restriction mapping of cosmid 13-6-4. In an effort to determine where on the cosmid the fragment that complements the *tutB16* mutation lies, deletions and subclones were constructed. All subclones were made in plasmid pRK415, a broad-host-range tetracycline resistance vector that can be conjugatively transferred into the T1 background in the same manner as pLAFR3 and is stably maintained in this background. Figure 2A shows a restriction map of cosmid 13-6-4. The relevant region of the cosmid is shown in more detail in Fig. 2B. The figure includes a number of subclones that were constructed in an effort to identify the region of the cosmid that contains the complementing gene.

Table 2 shows the results of complementation tests for the various subclones shown in Fig. 2B when mated into a T1 strain carrying the *tutB16* mutation. Complementation was assayed in three ways: (i) the ability to grow with toluene serving as the sole carbon source on solid media, (ii) the ability to metabolize toluene in the presence of pyruvic acid in liquid media, and (iii) the ability to produce the dead-end products benzylsuccinic acid and benzylfumaric acid from toluene in liquid media. The original clone and all complementing subclones were positive (i.e., behaved just as the wild-type strain) in all three assays.

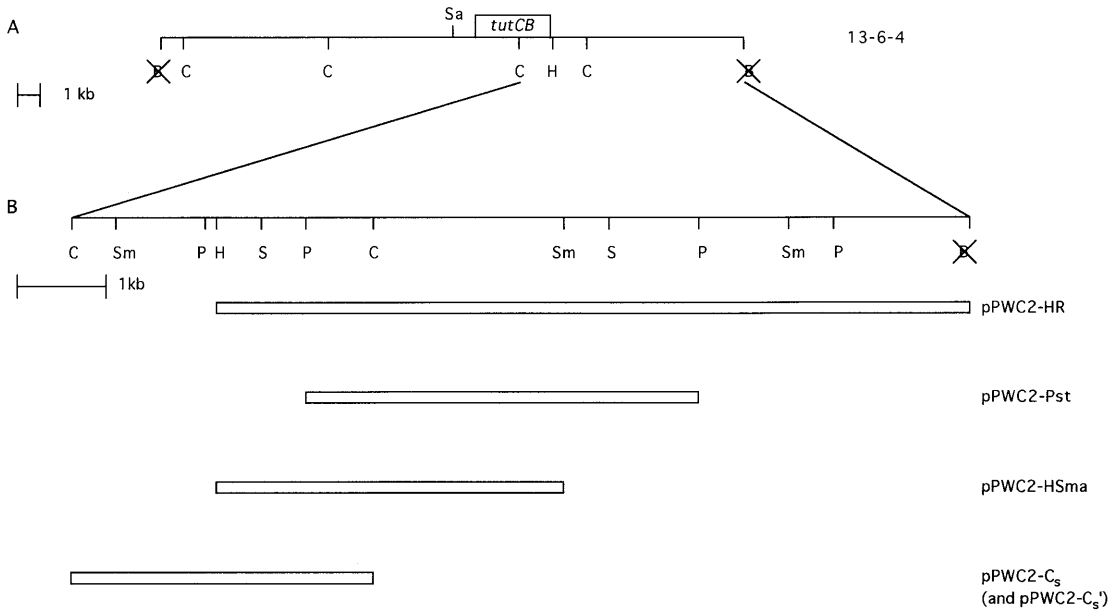

FIG. 2. (A) Restriction map of cosmid clone 13-6-4. (B) More detailed map of the complementing region and subclones. The location of the *tutCB* genes on 13-6-4 is identified with a box. Abbreviations: B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sa, *Sac*II; Sm, *Sma*I. Not all *Sac*II sites are shown.

As can be seen from Table 2, the small (3.3-kb) *Cla*I fragment of 13-6-4, when inserted into pRK415 in either orientation (Fig. 2B), is able to complement the *tutB16* mutation. Subclones constructed that do not contain this entire region do not complement this mutation. We conclude from these results that this 3.3-kb fragment is sufficient to replace the missing activity in the *tutB16* mutant strain.

Sequence analysis of the *tutCB* region. The complete nucleotide sequence of the 3.3-kb *Cla*I fragment of 13-6-4 (containing the *tutB* gene) was determined in both orientations. Analysis of this sequence revealed the presence of a second open reading frame (designated *tutC*) upstream of the *tutB* gene. As a result, the sequence was extended to a *Sac*II site about 3 kb upstream of the *Cla*I site. Figure 3 presents the complete 6,393-bp nucleotide sequence of the *tutCB* region. The protein translation of the two genes is presented below the DNA sequence in the figure. The TutC protein is 979 amino acids long with a calculated molecular mass of 108.0 kDa and a calculated pI of 5.2, while the TutB protein is 218 amino acids long with a calculated molecular mass of 24.3 kDa and a calculated pI of 7.9. Goldman-Engleman-Steitz hydropathicity analysis (9) failed to detect any membrane-spanning regions in either protein, but Kyte-Doolittle analysis (22) suggested two possible membrane-spanning regions in the TutC protein, amino acids 367 to 399 and 489 to 508 (data not shown). The translation of the *tutB* gene is shown as overlapping the sequence of the *tutC* gene by 13 nucleotides. This methionine was chosen as likely to be the first amino acid in the sequence based on the location of a potential Shine-Dalgarno sequence and protein similarity analysis (see below).

TABLE 2. Complementation of *tutB16* mutation by subclones

Subclone	Growth on toluene[a]	Toluene utilization[b]	Dead-end product accumulation[c]
None	−	−	−
13-6-4	+	+	+
pPWC2-HR	±	−	−
pPWC2-Pst	−	−	−
pPWC2-HSma	±	−	−
pPWC2-C_s	+	+	+
pPWC2-C_s′	+	+	+

[a] +, growth on toluene in 3 to 5 days; −, no growth in 3 to 5 days; ±, poor growth in 3 to 5 days.
[b] +, complete metabolism of 300 to 500 μM toluene in liquid medium in the presence of pyruvate in 3 to 4 days; −, metabolism of 0 to 100 μM in 3 to 4 days.
[c] +, accumulation of the dead-end compounds benzylsuccinic acid and benzylfumaric acid to the same extent as in the wild type in 3 to 4 days; −, accumulation of the dead-end compounds to the same extent as in the mutant strain *tutB16* in 3 to 4 days.

Sequence similarity analysis. The protein sequence of the *tutC* gene product was compared to the Swissprot protein database in an effort to identify other proteins with homologous sequences. The results of this analysis are presented in Fig. 4. The TutC protein shows significant sequence similarity to sensor members of the two-component family of signal transduction proteins (29), a set of bacterial regulatory proteins in which one member senses the environmental conditions of the microorganism and transmits a signal (via phosphorylation) to the other member (a DNA binding protein). The five proteins, all of which are sensor proteins, with the greatest sequence similarity to the *tutC* gene product are included in Fig. 4. These proteins (and their percent identity to the *tutC* gene product) are the products of the *nodV* gene of *Bradyrhizobium japonicum* (36%) (17); the *fixL* genes of *B. japonicum* (33%) (2), *Azorhizobium caulinodans* (30%) (20), and *Rhizobium meliloti* (30%) (8); and the *dctS* gene of *Rhodobacter capsulatus* (33%) (18).

In a similar manner, the sequence of the *tutB* gene product was compared to the Swissprot protein database in an effort to identify other proteins with homologous sequences. The results of this analysis are presented in Fig. 5. The TutB protein shows significant sequence similarity to DNA binding protein members of two-component sensor-regulator families (29). These proteins (and their percent identity to the *tutB* gene product) are the products of the *nodW* gene of *B. japonicum* (48%) (17); the *fixJ* genes of *B. japonicum* (38%) (2), *A. cau-*

FIG. 3. Nucleotide sequence of the *Sac*II-*Cla*I fragment of DNA containing the *tutCB* genes (see Fig. 2 for the location). Proposed amino acid translations are presented below the DNA sequence. Potential Shine-Dalgarno regions are underlined.

linodans (37%) (20), and *R. meliloti* (39%) (8); and the *dctR* gene of *R. capsulatus* (38%) (18). Because the similarity between these proteins and TutB extends nearly to the methionine that overlaps the *tutC* gene product, we propose that translation begins at this overlapping methionine, as shown in Fig. 3. Based on the results of the sequence similarity analysis and the previous result that the toluene utilization pathway of strain T1 is inducible (15), we propose that the *tutB* and *tutC* gene products are involved in the regulation of gene expression (specifically toluene-metabolic genes) in response to toluene.

DISCUSSION

Several pathways have been proposed for the anaerobic metabolism of toluene. Evans et al. proposed that strain T1 degrades toluene via phenylpropionyl-CoA and benzoyl-CoA (10) (Fig. 1). Other proposed pathways include metabolism to benzoic acid via benzyl alcohol and benzaldehyde for strain T and *Thauera aromatica* K172 (1, 33, 34) and via *p*-cresol, *p*-hydroxybenzyl alcohol, and *p*-hydroxybenzaldehyde to *p*-hydroxybenzoic acid for *Geobacter metallireducens* GS-15 (23). Chee-Sanford and coworkers have presented evidence that *Azoarcus tolulyticus* Tol-4 uses a pathway similar to that of strain T1, although a different dead-end product (*E*-phenylitaconic acid) has been identified in addition to benzylsuccinic acid (6, 27). Phenylitaconic acid has the same composition and molecular weight as benzylfumaric acid but differs in that the double bond is between the α and β carbons rather than the β and γ carbons on the side chain. Beller et al. also observed that the sulfate-reducing isolate PRTOL1 produced the dead-end products benzylsuccinic acid and benzylfumaric acid (or a closely related isomer) from toluene (3).

In an effort to better characterize the anaerobic pathway of toluene metabolism in strain T1, we have undertaken a genetic and molecular approach. We have isolated mutants that fall into two classes, one that fails to metabolize toluene and the other that metabolizes toluene but fails to use it as a growth substrate. This second class also fails to grow on benzoic acid, although this compound also is metabolized when pyruvic acid is provided as a growth substrate (data not shown).

Characterization of the mutants obtained in this work is a first step in the identification of the anaerobic toluene utilization pathway of strain T1. Our results are consistent with the proposed pathway shown in Fig. 1. The class of mutants blocked late in the pathway (*tutA*) metabolize toluene (but are unable to use it as a growth substrate) and produce the dead-

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2601 ATCGCAATTGACTCGGCAGATCATCGACAATCGCCAGGAGTTGCAGCGAAGCCATGATGCTTTGCAGGAATCTGAGTCCCGCTGGCGCGCAGTCTATGAG 2700
       S  Q  L  T  R  Q  I  I  D  N  R  Q  E  L  Q  R  S  H  D  A  L  Q  E  S  E  S  R  W  R  A  V  Y  E
2701 AATTCTGCTGCAGGTATTGTGTTGACAAATTTGGACGGCTTGATTTTGTCTGCGAATCAAGCATTTCAAAAAATGGTTGGCTATGCCGAGGATGAGTTGC 2800
       N  S  A  A  G  I  V  L  T  N  L  D  G  L  I  L  S  A  N  Q  A  F  Q  K  M  V  G  Y  A  E  D  E  L  R
2801 GGGTGATTGAAATATCGGATCTCGTCCCCGAACATGATCGCGAAAAAATCCGGTCGCGCGTTTCAAATCTGATCAGTGGCCGCGTCGACGACTATCAAGT 2900
       V  I  E  I  S  D  L  V  P  E  H  D  R  E  K  I  R  S  R  V  S  N  L  I  S  G  R  V  D  D  Y  Q  V
2901 GCAAAGGCAATGCCGACGAAAGGACGGCCGAATGATGTGGGCAAATGTGCGAGCATCGCTCATACCTGGGCTGGCCAATCAGTCTCCGATGGTTGTGAGA 3000
       Q  R  Q  C  R  R  K  D  G  R  M  M  W  A  N  V  R  A  S  L  I  P  G  L  A  N  Q  S  P  M  V  V  R
3001 ATTTTTGATGACATTACCGAAAAGATTCAGACTGAAGCTGAACTGGCAAGAGCAAGGGAAAAGTTGACCAGAGTCATGCGTGTTACCGCAATGGGAGAAT 3100
       I  F  D  D  I  T  E  K  I  Q  T  E  A  E  L  A  R  A  R  E  K  L  T  R  V  M  R  V  T  A  M  G  E  L
3101 TGGCGGCATCGATTGCTCATGAGTTGAATCAACCGCTTGCCGCCATTGTTACCAATGGTCATGCATCATTACGCTGGCTTGGCTCCGAGCCTTGTAATCT 3200
       A  A  S  I  A  H  E  L  N  Q  P  L  A  A  I  V  T  N  G  H  A  S  L  R  W  L  G  S  E  P  C  N  L
3201 ATTGGAAGCCGTCGAAGCAGTGCGAAGAATCATCCATGATGCTAATCGCGCGAGTGAAATAATCAAACGGATCCGTGGCTTTCTTCAGCGGGGGGAGGGG 3300
       L  E  A  V  E  A  V  R  R  I  I  H  D  A  N  R  A  S  E  I  I  K  R  I  R  G  F  L  Q  R  G  E  G
3301 AGGCGCTCGGCAGTGGATATTTTTCAGGTTGTTGCGGATGTGGCTGCGATTGTCAGCGATATGGCGCGCAGTCATTGCATTGATATGCGTTATCAAGCAG 3400
       R  R  S  A  V  D  I  F  Q  V  V  A  D  V  A  A  I  V  S  D  M  A  R  S  H  C  I  D  M  R  Y  Q  A  V
3401 TCGGTCAATTGTCGCTAGTGATTGCGGATAAGGTCCAGTTGCAACAGGTTATTCTGAATTTGTGCATCAATGGCATAGAATCCATTGTTGGCGGAAACTC 3500
       G  Q  L  S  L  V  I  A  D  K  V  Q  L  Q  Q  V  I  L  N  L  C  I  N  G  I  E  S  I  V  G  G  N  S
3501 CGAACGAGGCGAACTTTCAATTACCGTTACCCAGTCCGATAAAAGATTCTTGACCGTCAGCGTACATGATTCCGGCCCGGGCCTTGCACCTGGCGAGGCG 3600
       E  R  G  E  L  S  I  T  V  T  Q  S  D  K  R  F  L  T  V  S  V  H  D  S  G  P  G  L  A  P  G  E  A
3601 GAAAACGTGTTTGATGCGTTCTATACGAGCAAGGTGGAGGGGCTTGGCATGGGGCTCGCCATCAGTCGCTCTATCATTGAGGCGCATGGTGGGCGCCTTG 3700
       E  N  V  F  D  A  F  Y  T  S  K  V  E  G  L  G  M  G  L  A  I  S  R  S  I  I  E  A  H  G  G  R  L  D
3701 ATGTTCTGTCCCCTTCCACGGAGGGGGGATGCACGTTCTGTTTCACGTTGCCTACGGAGGAGATGGCTAGCCCATGTGCCCCACAATAGATGCATCGACT 3800
       V  L  S  P  S  T  E  G  G  C  T  F  C  F  T  L  P  T  E  E  M  A  S  P  C  A  P  Q  *
                                                                                M  C  P  T  I  D  A  S  T
3801 GTTTATCTGGTGGACGACGATCGCTCCATGCGTGACGCAATTTCCAGCTTGGTTCGATCGGTCGGCCTCAATGTGGAGACATTTGCGTCTGCAAGTGAGT 3900
       V  Y  L  V  D  D  D  R  S  M  R  D  A  I  S  S  L  V  R  S  V  G  L  N  V  E  T  F  A  S  A  S  E  F
3901 TCTTGGAGCACGCTCGTTCGGAAGCATGTGCCTGCTTGGTTCTTGATGTTCGGATGCCACGCATGAGCGGTTTTGATCTTCAGCATGCGTTAAGCAAAAA 4000
       L  E  H  A  R  S  E  A  C  A  C  L  V  L  D  V  R  M  P  R  M  S  G  F  D  L  Q  H  A  L  S  K  N
4001 TGGTGTCGATATTCCAATCATCTTTATTACCGGCCATGGTGATATCCCCATGGCGGTTCGCGCCATCAAGTCGGGTGCCCTAGAATTTCTTCCAAAGCCT 4100
       G  V  D  I  P  I  I  F  I  T  G  H  G  D  I  P  M  A  V  R  A  I  K  S  G  A  L  E  F  L  P  K  P
4101 TTTCGTGCTGAAGAACTGCTCGAAGCAATCAACAGGGCTCTGAATATCGATCAGGAGGCTCGGGAGTACAAGGCGGAGCTGGATAAGATATTGAAGAAAT 4200
       F  R  A  E  E  L  L  E  A  I  N  R  A  L  N  I  D  Q  E  A  R  E  Y  K  A  E  L  D  K  I  L  K  K  Y
4201 ATGAGGGGCTTACAGATCGAGAAAAGGAGGTATTTCCCCTTATTGCCCAGGGCTTGTTGAACAAGCAGATTGCCGGATATCTCGGAATTACTGAGGTCAC 4300
       E  G  L  T  D  R  E  K  E  V  F  P  L  I  A  Q  G  L  L  N  K  Q  I  A  G  Y  L  G  I  T  E  V  T
4301 CATAAAGGTTCATCGTCATAATATTACGAGAAAAATGGGGGTCCGGACACTGGCTAATCTGGTGCGACTTTACGAGAAGTTAAAGAATGCTGGGCTGATC 4400
       I  K  V  H  R  H  N  I  T  R  K  M  G  V  R  T  L  A  N  L  V  R  L  Y  E  K  L  K  N  A  G  L  I
4401 GAAAAAAAGAACGGAAATCTATCGGGATGAAGAGCCGCGACTGGAACCCTTCAGGCTCTTGGCGGCCACGCTGTAGGAACGCTATCGCCTACCTGCGAAT 4500
       E  K  K  N  G  N  L  S  G  *
4501 GTCTAAACTCACTGAAACGGCATAGAGTTCAAAGCAAGAACTTAGCAAAATGGATTTGCCTAACAGTTGATTGTAGAAATAATTTTTTATTGATTAATGA 4600
4601 TCGGTTGATTGTTGCTGCAGTCCTGGGAGGGAAAGCCATTCACAAGCACTACAATGACTGCTGCTGCGCATCGCAAAATGTATCAAGTCGCCGGTGGACC 4700
4701 TCAGTCCAAGCTTGCTCATGATGCGCCCGCGGTGAGCTTCCACAGTCTTATGGCTGATGCCCAGCAGCTTGGCGATTTCCTTGCTGCTGTTGCCGCAAAC 4800
4801 CACCTTGTCGAGAATCTCCATTTCGCGCTGTGACAGGGCGGCAAGAAGATCGGCACGCTCCTGCTTTTCGCGCTGCTTATTCGTCATCTCCTTGCTGAAG 4900
4901 GCAAGGGCGGCGTTTACAGCATCAAGCAGATCCTGGTTGCGATAGGGTTTTTGGAGAAAGTCGAACGCGCCTTTCTTTACCGCTTGAGCTCCCATCTGGG 5000
5001 CATCGCCGTAGGCGGAGAGAAAGATGATCGGCAGCTTGAAGCCTCTGTCACACAGCGCTTGTTGCAACTGTAGGCCGCTGACATTTTGCATTCGAACATC 5100
5101 CAGAATCACACAGCCGTAGGTGCATGAAATATCGGCATCAAGAAAATGCTTTGCGGATTCGTATGCCTTGACGTTTAGTGAAATCGAGTTGAGGAGCCAC 5200
5201 GTCAGTGAGTCCCTGACCGACGCTTCGTCGTCGACGACGAATACAGTTGGGGCGTTTCCAGATTTTTCGATTTGCATGAGGTGCTCCTTTGTGCGGTGCT 5300
5301 ATGACAAACGTACGGTCTATATGGCATCGCTAATCTGAATATCTCTTACATTGGCAAGGTAAACTGAAACCTCGATCCGCATCCTTCGGTCTTCGAAAAT 5400
5401 GAAAGTTCGCCCCCATGTGATTCGATGATCGAGCGGCAGATCGTCAATCCTATTCCAAGTCCGTCTGGCTTGGTTGTGAAATAGGGTTTAAAGACGCGTT 5500
5501 TTGCTGTTCGGCTTTCGACGCCGGTCCCATAGTCTTGTACAAACACCTTCATTGACCTTCCCTCTGCAATGATTTCGCTACCGATCAGCAGCACGCGAGA 5600
5601 GTCCGGTTCCATTTCTGACATGGCCTCAATGCCATTCTTGATTAGATTGAATAAGACCTGCTGGATCTCGATTTTGCAGAGAGGGATGAGTGGAGGGTCG 5700
5701 GGCATTAACTGCAGATTCACCGTAGCGTTATGTCGATGGATCTCGAAACTTAGAAATGAAAGAGCGTCCTGGATGACGTCGTTGATGTTTTCCAATAGCC 5800
5801 TTTCTGGCTTGTGTTTGCAGACGAAATCCTTGACACGCCGCAGGATCTCACCCGCTTGATCTAGATGGGTGATTGCTAACCCGAGTGAGTGTGATATCTC 5900
5901 CTCGACTCCGGGCACATGTTCAAGCCGTAGCCGGCAGCCCTGGAGATAGTTCACGGCGGAAACCAGGGGTTGGCCCATCTGGTGTGCAAGCGCTGCGGCC 6000
6001 ATTCCGGCCATCGCGTTGATGCGTCCGAGTCGAGTGAGTTCTGCGTGACGGAGCCTTTCCAATTCCTCTATCCGCTTGCGGTAGGTGATATCTGCGAAGG 6100
6101 CGGCCACAACTATTTTCTCATCCTTGATCTCGAGCAAGGATGAGCTGACGCTGAGCCATCGCGTCTTGTGATTTTCTTCGTCGCACATGCCGACTTCGAG 6200
6201 GCTTCTGACCGAACTTTTCTGGAGGTCGTGAATCCGCCAAGGCAATCGCTTTTTCCAGATATTTGTCCCGTCATTGAGAAAGAACCGTTGCGGTAATTGC 6300
6301 TGCCAACTCATGGGCGTCCCTTCCTGTGTTCCCATCAGTTCAGAAAACTGGCAGTTTTCCTCGAGAATCCTGCCGCGGCTATCAGTAATCGAT 6393
```

FIG. 3—*Continued.*

end products benzylsuccinic acid and benzylfumaric acid, as would be predicted from the pathway. Similarly, in agreement with the predicted result, mutants in the *tutB* class are unable to metabolize toluene and fail to produce the dead-end products. This observation indicates that there is a common enzymatic step in the two branches of the toluene metabolic pathway of strain T1. Since the wild-type strain does not produce the dead-end products from phenylpropionic acid (15), our evidence supports a pathway that branches at the earliest point, as presented in Fig. 1, which is in contrast to the pathway proposed for *A. tolulyticus* Tol-4 (6). Further characterization of the *tutB* class of mutants and cloning and characterization of additional genes involved in the anaerobic toluene utilization pathway of strain T1 will enable us to establish the mechanism of this metabolic pathway.

The cloned *Cla*I fragment of about 3.3 kb (containing the complete *tutB* gene and a fraction of the *tutC* gene) that fully complements the *tutB16* mutation carries all the information necessary to restore the ability to utilize toluene to this mutant strain. The subclone complements the mutation when inserted into the pRK415 vector in either orientation. This strongly suggests that the subclone provides all the *cis*-acting factors

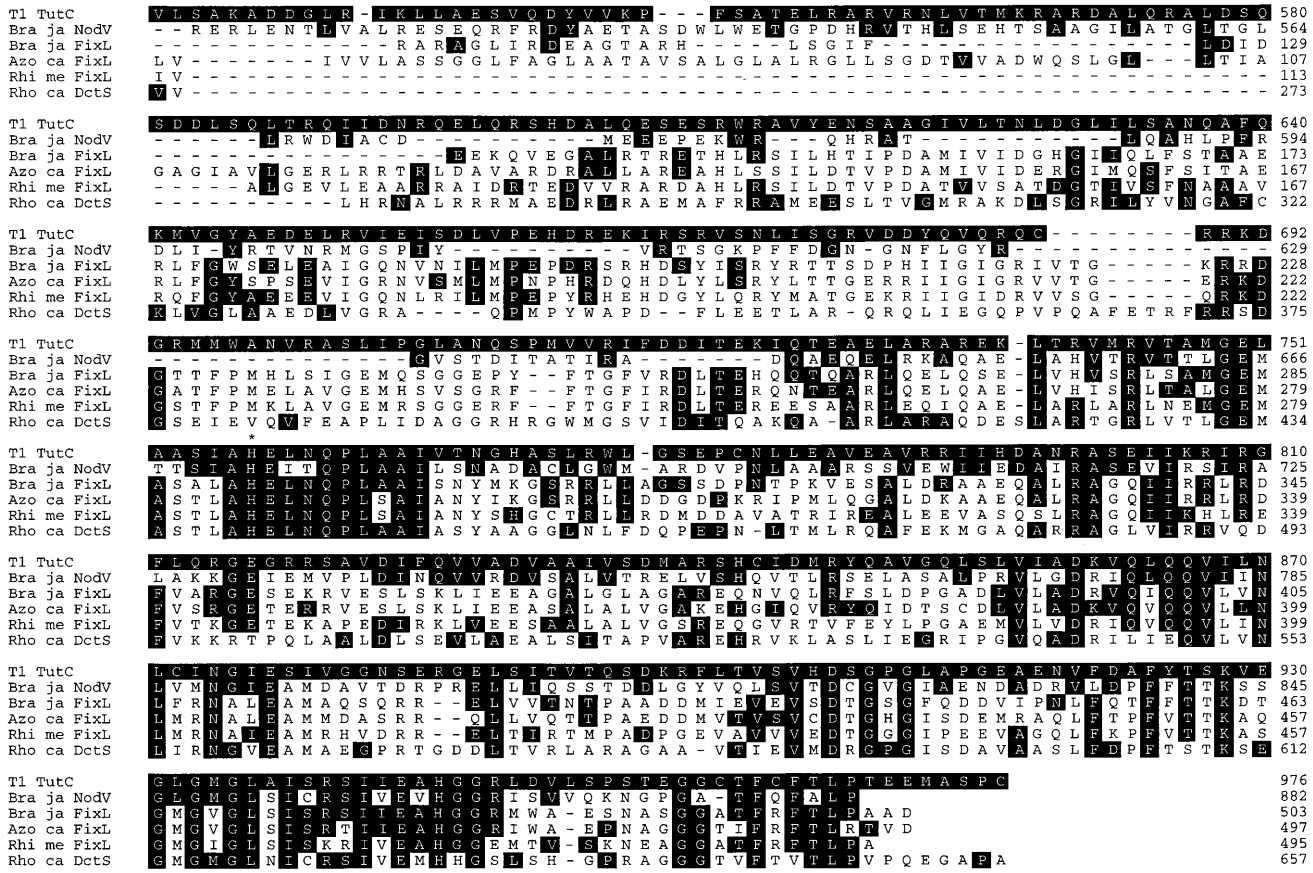

FIG. 4. Comparison of the predicted amino acid sequence of the *tutC* gene product (452 of 979 amino acids) to the predicted sequence of the *nodV* gene product of *B. japonicum* (376 of 889 amino acids) (17); the *fixL* gene products of *B. japonicum* (399 of 505 amino acids) (2), *A. caulinodans* (440 of 504 amino acids) (20), and *R. meliloti* (385 of 505 amino acids) (8); and the *dctS* gene product of *R. capsulatus* (387 of 657 amino acids) (18). Only the portion of the proteins containing the major region of sequence similarity is included in the figure. The conserved histidine residue that is proposed to autophosphorylate is shown with an asterisk above it. Amino acids identical to the *tutC* translation are shaded.

necessary for gene expression and the vector does not provide any elements essential for expression of the insert. In addition, although 13-6-4 complements the *tutB17* and *tutB21* mutations, the construct containing only the 3.3-kb *Cla*I fragment (pPWC2-C_s) was not able to complement these other mutants, indicating that the *tutB* class of mutants consists of more than one gene (data not shown). These other mutants are being examined in the hope of isolating the gene whose product carries out the first step in the toluene metabolic pathway.

DNA sequence analysis of the 3.3-kb *Cla*I fragment has

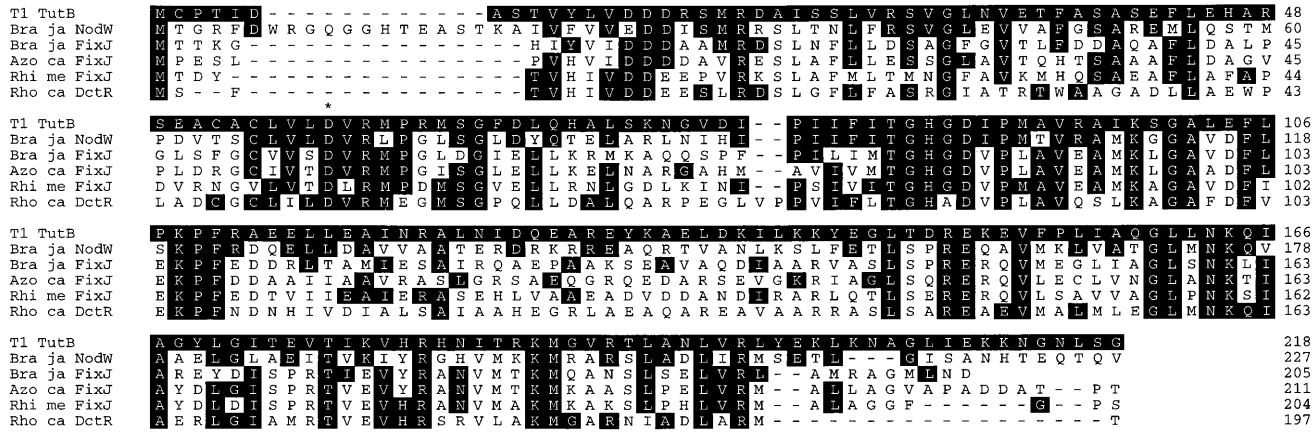

FIG. 5. Comparison of the entire predicted amino acid sequence of the *tutB* gene product to the entire predicted sequence of the *nodW* gene product of *B. japonicum* (17); the *fixJ* gene products of *B. japonicum* (2), *A. caulinodans* (20), and *R. meliloti* (8); and the *dctR* gene product of *R. capsulatus* (18). The conserved aspartic acid residue that is proposed to be phosphorylated is shown with an asterisk above it. Amino acids identical to the *tutB* translation are shaded.

identified an open reading frame whose product has homology to the *nodW* gene product of *B. japonicum* (17) and other proteins presented in Fig. 5. All of these proteins have been identified as DNA binding regulatory proteins and members of the two-component family of signal transduction proteins (29). They all have phosphorylation sites at a conserved aspartic acid residue. The *tutB* gene product also has an aspartic acid residue in the analogous location, at amino acid 58.

Additional DNA sequence analysis has identified a second open reading frame, *tutC*, upstream of the *tutB* gene. The product of this open reading frame has homology to the *nodV* gene product of *B. japonicum* (17) and other proteins presented in Fig. 4. These gene products are proposed to serve as the sensor protein in the two-component regulatory system (29). In their role as sensor proteins, they must autophosphorylate and then transfer the phosphate to the DNA binding protein. The site of autophosphorylation is a histidine residue that is conserved in all the systems. The *tutC* gene product has a histidine residue in the analogous location at amino acid 757. As can also be seen in Fig. 4, the homology of the sensor proteins extends only about 400 amino acids. This region is proposed to be the transmitter domain, the part of the protein that sends the regulatory signal to the DNA binding protein. The remainder of the protein presumably serves to detect the signal from the environment and would not be expected to be conserved across the different systems.

The proteins that have the greatest similarity to the *tutCB* gene products appear to regulate a diverse set of genes. Both FixL and FixJ from *R. meliloti* and from *A. caulinodans* regulate genes involved in nitrogen fixation (8, 20), while FixL and FixJ from *B. japonicum* are proposed to regulate anaerobic respiratory genes (2). The *nodVW* gene products of *B. japonicum* play a role in the nodulation process (17), while the *dctSR* gene products of *R. capsulatus* serve as regulators of C_4-dicarboxylate transport (18). It is apparent that these genes function in a similar manner, but the classes of genes they regulate have little in common.

Although the toluene metabolic pathway of a number of aerobic strains has been studied extensively and genes involved in the metabolism and regulation of the five pathways have been cloned, this is, to our knowledge, the first report of the cloning and sequencing of genes from an anaerobic strain involved in the metabolism of toluene. Every pathway so far proposed for anaerobic toluene metabolism differs from the aerobic pathways currently studied. Isolation of mutants presented in this paper and the cloning of proposed regulatory genes involved in the toluene utilization pathway of strain T1 are the first steps to a better understanding of the mechanism and regulation of the enzymatic reactions of the *tut* pathway in this denitrifying strain and, perhaps, the anaerobic toluene metabolic pathways of other strains.

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