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Aerobic and Anaerobic Toluene Degradation by a Newly Isolated Denitrifying Bacterium, *Thauera* sp. Strain DNT-1

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A newly isolated denitrifying bacterium, *Thauera* sp. strain DNT-1, grew on toluene as the sole carbon and energy source under both aerobic and anaerobic conditions. When this strain was cultivated under oxygen-limiting conditions with nitrate, first toluene was degraded as oxygen was consumed, while later toluene was degraded as nitrate was reduced. Biochemical observations indicated that initial degradation of toluene occurred through a dioxygenase-mediated pathway and the benzylsuccinate pathway under aerobic and denitrifying conditions, respectively. Homologous genes for toluene dioxygenase (*tod*) and benzylsuccinate synthase (*bss*), which are the key enzymes in aerobic and anaerobic toluene degradation, respectively, were cloned from genomic DNA of strain DNT-1. The results of Northern blot analyses and real-time quantitative reverse transcriptase PCR suggested that transcription of both sets of genes was induced by toluene. In addition, the *tod* genes were induced under aerobic conditions, whereas the *bss* genes were induced under both aerobic and anaerobic conditions. On the basis of these results, it is concluded that strain DNT-1 modulates the expression of two different initial pathways of toluene degradation according to the availability of oxygen in the environment.

Benzene, toluene, ethylbenzene, and xylenes (BTEX) are one of the most common groups of groundwater contaminants. Of these contaminants, toluene is degraded by many strains of aerobic bacteria, and five different aerobic toluene degradation pathways have been identified. *Burkholderia cepacia* G4, *Ralstonia pickettii* PKO1, and *Pseudomonas mendocina* KR1 first oxidize toluene using specific monooxygenases to form *o*-, *m*-, and *p*-cresol, respectively (34, 35, 52). The cresols formed by strains G4 and PKO1 undergo a second monooxygenation to form 3-methylcatechol, which is then degraded by a *meta* ring fission pathway (35, 45). In strain KR1, the methyl group of *p*-cresol is oxidized, and the resulting 4-hydroxybenzoate is degraded by an *ortho* cleavage pathway (51). On the other hand, *Pseudomonas putida* mt-2 oxidizes the methyl group of toluene to form benzoic acid, which is further metabolized through a *meta* cleavage pathway via catechol (4). *P. putida* F1 carries the chromosomally encoded *tod* pathway. Toluene dioxygenase (TodC1C2BA) oxidizes toluene to *cis*-toluene dihydrodiol. Then, toluene dihydrodiol dehydrogenase (TodD) transforms the dihydrodiol to 3-methylcatechol, which is cleaved by the *meta* fission enzyme 3-methylcatechol 2,3-dioxygenase (TodE) (20).

The anaerobic pathway for toluene degradation has been elucidated primarily in three denitrifying strains, *Thauera aromatica* K172, *T. aromatica* T1, and *Azoarcus* sp. strain T. In all strains, the initial reaction is the addition of fumarate to the methyl group of toluene, a reaction catalyzed by benzylsuccinate synthase. The product of the reaction, benzylsuccinate, is converted to phenylitaconyl coenzyme A (phenylitaconyl-CoA) by CoA transfer with succinyl-CoA and further metabolized through a series of β oxidation-like reactions to benzoyl-CoA. Benzoyl-CoA is the central metabolite of the anaerobic degradation of aromatic compounds and is dearomatized by benzoyl-CoA reductase prior to ring cleavage (8, 48).

The strains described above are able to degrade toluene under either aerobic or anaerobic conditions, but not both. A few studies have reported the identification of denitrifying strains that can grow on toluene under both aerobic and anaerobic conditions (9, 18, 25, 47), but details of the pathways used are not known. Another aromatic compound, 4-hydroxybenzoate, supports the growth of *Rhodopseudomonas palustris* under both aerobic and anaerobic conditions. Anaerobic degradation is initiated by CoA thioesterification of the carboxyl group, and the product, 4-hydroxybenzoyl-CoA, is dehydroxylated to form benzoyl-CoA, which is then dearomatized and the ring is cleaved (21). In the presence of oxygen, 4-hydroxybenzoate is oxidized to protocatechuate and further degraded by the *meta* fission pathway (22). The denitrifying bacterium *Azoarcus evansii* initiates degradation of 2-aminobenzoate, benzoate, and phenylacetate by CoA thioesterification under both aerobic and anaerobic conditions, and different sets of enzymes are used (2, 10, 19, 33, 40, 43). Recent study revealed that in the case of benzoate degradation by *T. aromatica* K172, common benzoyl-CoA ligase initiates both aerobic and anaerobic degradation of benzoate by CoA thioesterification (42).

In this study, *Thauera* sp. strain DNT-1, a strain that degrades toluene under both aerobic and anaerobic conditions, was isolated. The initial toluene degradation pathways present in this strain were characterized, and the control of transcrip-

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tion of the putative catabolic genes was investigated. This is the first report confirming that two different initial pathways for toluene degradation in a single bacterial strain are controlled in response to the oxygen concentration.

MATERIALS AND METHODS

Enrichment and isolation. The composition and preparation of the enrichment medium were as reported by Tschech and Fuchs (49). Sources of microorganisms were incubated under N_2 atmosphere at 30°C in this medium (9 ml) using anaerobic culture tubes (Bellco Glass Inc., Vineland, N.J.), with 1 ml of light white mineral oil (Sigma Chemical Co., St. Louis, Mo.) containing 0.1% (vol/vol) toluene layered as the "carrier phase" of the sole carbon and energy source (39). After several transfers, bacteria were isolated by repeated plating on solidified enrichment medium with toluene vapor supplied as 1% (vol/vol) toluene in hexadecane (17) or on nutrient agar medium incubated either aerobically or anaerobically using an anaerobic jar (Becton Dickinson and Company, Franklin Lakes, N.J.). *Thauera* sp. strain DNT-1 was isolated from one of the enrichment cultures inoculated with anaerobic sludge from a wastewater treatment plant.

Media and growth conditions. First, the culture conditions were examined. The composition of the growth medium used follows: 1.6 g of Na_2HPO_4, 1.0 g of KH_2PO_4, 0.5 g of NH_4Cl, 0.06 g of K_2SO_4, 0.025 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.42 g of $NaHCO_3$, 15 mg of EDTA · 2Na, 1.5 mg of $FeSO_4 \cdot 7H_2O$, 20 mg of vitamin B_{12}, and 10 mg of D-biotin (all in 1 liter of distilled water) (pH 7.0 to 7.1). Toluene (0.5 mM) was added as the sole carbon source, and KNO_3 (5 mM) was added when required. *Thauera* sp. strain DNT-1 was routinely cultivated aerobically on nutrient agar medium containing 5 mM KNO_3 or anaerobically on toluene in growth medium containing 5 mM KNO_3 using anaerobic culture tubes at 30°C without shaking.

For cultivation with different oxygen concentrations, growth medium (40 ml) in 50-ml serum bottles was equilibrated in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, Mich.) for more than 2 days and was inoculated with *Thauera* sp. strain DNT-1 grown anaerobically on toluene. The gas phase (30 ml) inside the bottles was a gas mixture containing N_2 (95%) and H_2 (5%) for anaerobic cultivation or was exchanged with air for aerobic cultivation or exchanged with a gas mixture containing N_2 (98%) and O_2 (2%) for oxygen-limiting cultivation. Each culture was incubated at 30°C with reciprocal shaking.

Chemical analysis. Toluene concentrations were determined by gas chromatography with a flame ionization detector, using a G-100 column (40 m by 1.2 mm; Chemicals Inspection and Testing Institute, Tokyo, Japan), helium as the carrier gas (60 kPa), and column, injector, and detector temperatures of 70, 120, and 200°C, respectively. The concentrations of toluene metabolites, nitrate, and nitrite were analyzed by high-performance liquid chromatography performed with a UV detector (model LC-10A type PIA; Shimadzu Corp., Kyoto, Japan). A metabolite was detected at 275 nm on a Wakosil 5C18 HG column (150 by 4.6 mm; Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 30°C. The solvent system used was 0.1% H_3PO_4-methanol (50:50), with a flow rate of 1.0 ml/min. The nitrate and nitrite concentrations were measured at a wavelength of 210 nm on a TSKgel IC-Anion PW column (50 by 4.6 mm; Tosoh Corp., Tokyo, Japan) at 40°C. The eluent used contained 1.3 mM potassium gluconate, 1.3 mM sodium tetraborate, 30 mM borate, 10% (vol/vol) acetonitrile, and 0.5% (vol/vol) glycerol, and the flow rate was 1.2 ml/min. The culture fluid was centrifuged at 15,000 × *g* for 10 min, and 10 μl of the diluted supernatant was analyzed. Liquid chromatography and mass spectrometry (LC/MS) analysis of the metabolite was performed by Thermoquest K. K. (Osaka, Japan). The molecular oxygen concentration was measured by gas chromatography with a thermal conductivity detector (current, 100 mA), using a molecular sieve 13X 60/80 mesh column (2.1 m by 2.6 mm; GL Sciences Inc., Tokyo, Japan), helium as a carrier gas (40 ml/min), and column, injector, and detector temperatures of 50°C.

Analysis of 16S rDNA. 16S ribosomal DNA (rDNA) was selectively amplified by PCR from a cell lysate of the strain and sequenced as previously described (46). Taxonomic studies were also performed at the National Collections of Industrial, Food and Marine Bacteria (NCIMB) (Aberdeen, Scotland).

Southern blot analysis and gene cloning. Primers used in this study are listed in Table 1. Plasmid pJHFC1C2 (54), carrying the toluene dioxygenase genes *todC1C2BA* from *P. putida* F1 (26), was restricted with *Sac*I and *Hin*dIII, and the fragment, named todC1p, encoding the large subunit of toluene dioxygenase corresponding to positions 103 to 2186 of the gene (accession no. J04996) was isolated. The oligonucleotide primers bssAf and bssAr, derived from the nucleotide sequence of *bssA* from *T. aromatica* K172 (accession no. AJ001848) were used for PCR amplification of an internal fragment of the putative benzylsuccinate synthase gene of *Thauera* sp. strain DNT-1. The 1.0-kb PCR product, called bssAp, was subsequently cloned and sequenced. The predicted amino acid sequence of bssAp showed homology to that of *bssA* (data not shown). TodC1p and bssAp were used as probes for Southern blotting (41) of strain DNT-1 chromosomal DNA, which was extracted by the standard procedure (29) from cells grown aerobically on 2× YT medium (41) supplemented with $FeSO_4$ and vitamins as the growth medium. Hybridization was performed by using Alkphos DIRECT (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Single signals were detected among the *Pst*I fragments probed by todC1p and *Bam*HI fragments and *Sac*I fragments probed by bssAp, with sizes of 4.3, 6.5, and 6.0 kb, respectively. Restriction fragments of the corresponding sizes were ligated into pBluescript II SK$^+$ (Stratagene, La Jolla, Calif.), and libraries of chromosomal DNA of strain DNT-1 were constructed. Colony blots containing these libraries were probed by todC1p or bssAp, and each fragment was cloned and named pTD1, pTD18, and pTD21, respectively. Their sequences were determined on both strands. Homology searches of the deduced amino acid sequences were conducted using the FASTA program (37) through the DDBJ website (http://www.ddbj.nig.ac.jp/Welcome-j.html).

TABLE 1. Primers used in this study

Primer	Nucleotides	Sequence
bssAf	8009–8027[a]	TGGGTCAACGTGCTGTGCA
bssAr	9034–9017[a]	GCCGGATACGCGCACGAT
todA-RT	3483–3463[b]	GTTACGGTTCGCGCCGCATTC
todAf	2879–2899[b]	CTTAGCGGTGAGCGCAGTGTG
todAr	3208–3188[b]	CCACGATGTAGCGACTTGGGG
bssA-RT	2318–2298[c]	CTGGCGCTTCGGATCGGTTTC
bssAf-2	1846–1866[c]	CGCTGTATCCCGAACTGTCCC
bssAr-2	2166–2146[c]	AGGGGAAGGTGGACATATCGC

[a] The positions correspond to the positions of the nucleotide sequence deposited in GenBank/EMBL/DDBJ Nucleotide Sequence Data Library under accession number AJ001848.

[b] The positions correspond to the positions of the nucleotide sequence deposited in GenBank/EMBL/DDBJ Nucleotide Sequence Data Library under accession number AB066264.

[c] The positions correspond to the positions of the nucleotide sequence deposited in GenBank/EMBL/DDBJ Nucleotide Sequence Data Library under accession number AB066263.

Northern blot analysis. Total RNA of *Thauera* sp. strain DNT-1 was extracted from cells at early logarithmic phase and grown under aerobic and anaerobic conditions with either toluene or succinate as the sole carbon source using the NucleoSpin RNA II Mini kit (MACHEREY-NAGEL, Düren, Germany). Portions of the putative *todA* and *bssA* genes were obtained by PCR with specific primers todAf and todAr and specific primers bssAf-2 and bssAr-2, respectively, and were used as DNA probes for Northern blot hybridizations (41) by using Alkphos DIRECT (Amersham Pharmacia Biotech).

Real-time quantitative reverse transcriptase PCR (QRT-PCR). The same batch of extracted RNA as used for Northern blot analyses was used for the following experiments. cDNA was synthesized in a final volume of 20 μl that included 5 μg of total RNA, by using ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. todA-RT and bssA-RT (5 pmol each) were used as primers to amplify cDNA corresponding to the putative *todA* and *bssA* genes, respectively. After reverse transcription for 50 min at 42°C, the samples were heated for 5 min at 99°C to terminate the reaction, and 0.5 μl of RNase H was added. Real-time quantitative PCR was performed in a final volume of 20 μl in glass capillary tubes in a LightCycler Instrument (Roche Diagnostics, Basel, Switzerland) (53). The PCR master mix contained 3 mM $MgCl_2$, 0.5 pmol of primers, and 1× LightCycler-DNA Master SYBR green I (Roche Diagnostics). Primers todAf and todAr were used to detect the *todA* transcript, and primers bssAf-2 and bssAr-2 were used to detect the *bssA* transcript. Into each capillary tube, 17 μl of master mix and 1 μl of cDNA were loaded. Sealed capillary tubes were centrifuged prior to placement into the LightCycler carousel. PCR amplification was performed as follows: (i) an initial denaturation at 95°C for 5 min, (ii) 50 cycles, with 1 cycle consisting of denaturation at 95°C for 10 s, annealing at 60°C for 5 s, and elongation at 72°C for 16 s (temperature transition, 20°C/s). Amplicon specificity was verified by melting-curve analyses conducted at 65 to 95°C (temperature transition, 0.1°C/s) with stepwise fluorescence acquisition and by ethidium bromide staining on 2% agarose gels. No fluorescence was detected from real-time Q-PCR amplification without a template.

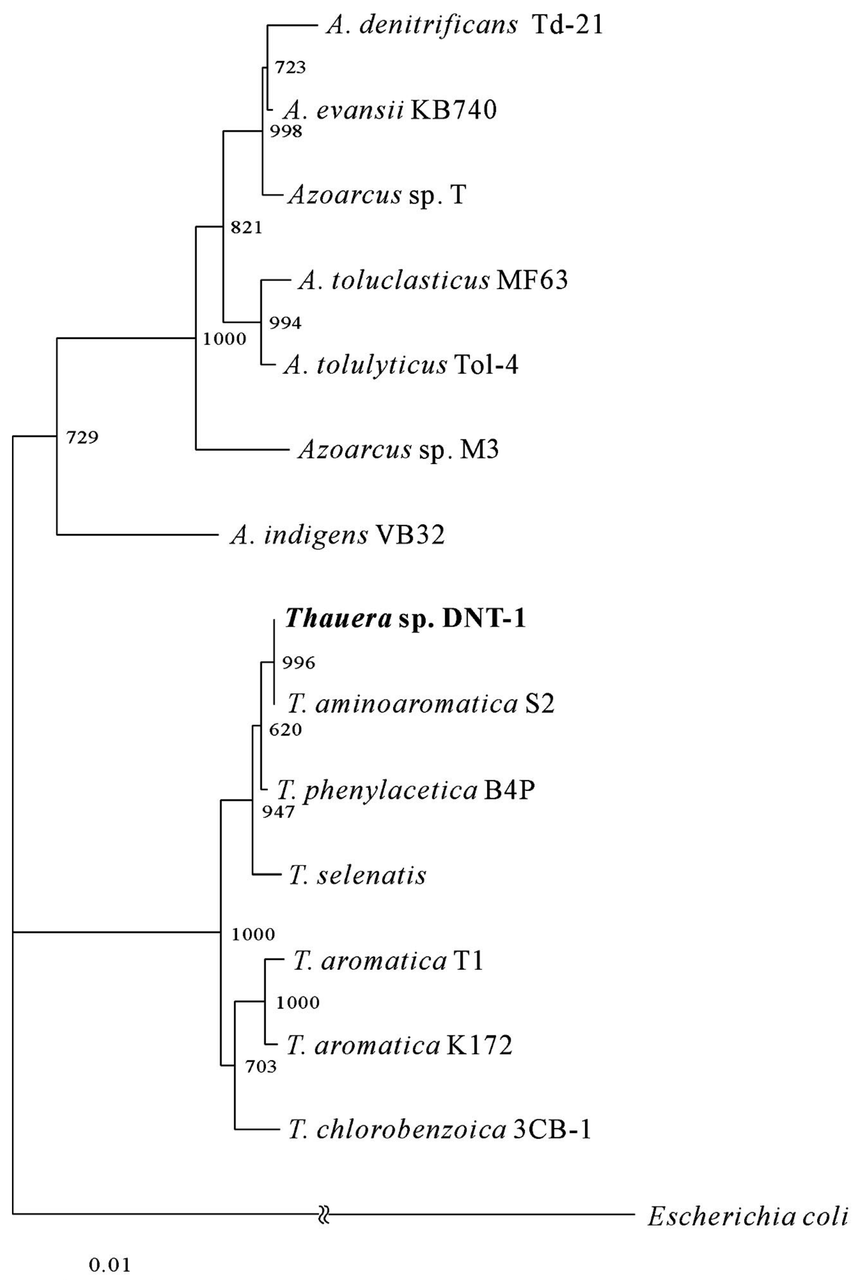


FIG. 1. Phylogenetic position of *Thauera* sp. strain DNT-1 among related aromatic compound-degrading *Thauera* and *Azoarcus* strains based on 16S rDNA sequence comparisons. The numbers are bootstrap values for branches based on 1,000 replicates. The bar shows 1 nucleotide substitution per 10,000 nucleotides.

Nucleotide sequence accession numbers. The nucleotide sequences of 16S rDNA and the *bss* and *tod* genes have been deposited in the DDBJ database under accession numbers AB066262, AB066263, and AB066264, respectively.

RESULTS

Isolation and identification of *Thauera* sp. strain DNT-1. A toluene-degrading denitrifying bacterium, strain DNT-1, was isolated from anaerobic sludge from a wastewater treatment plant. The bacterial cells were short rods, 2 to 2.5 μm in length and 1.5 μm in diameter, and were gram negative, catalase negative, and oxidase positive. 16S rDNA sequencing and taxonomic analyses revealed that this strain belongs to the genus *Thauera* and is closely related to *T. aminoaromatica* S2 (Fig. 1). Strain DNT-1 was able to grow on toluene, benzaldehyde, and benzoate under both aerobic and denitrifying conditions. Ethylbenzene, *n*- and *iso*-propylbenzene, and biphenyl supported aerobic growth. *p*-Cresol and *p*-hydroxybenzoate supported growth under anaerobic denitrifying conditions. Phenol, *o*- and *m*-cresols, and xylenes did not support growth under either conditions. It is noteworthy that the culture medium transiently turned yellow during aerobic growth on biphenyl, suggesting the presence of a *meta* ring cleavage dioxygenase in this

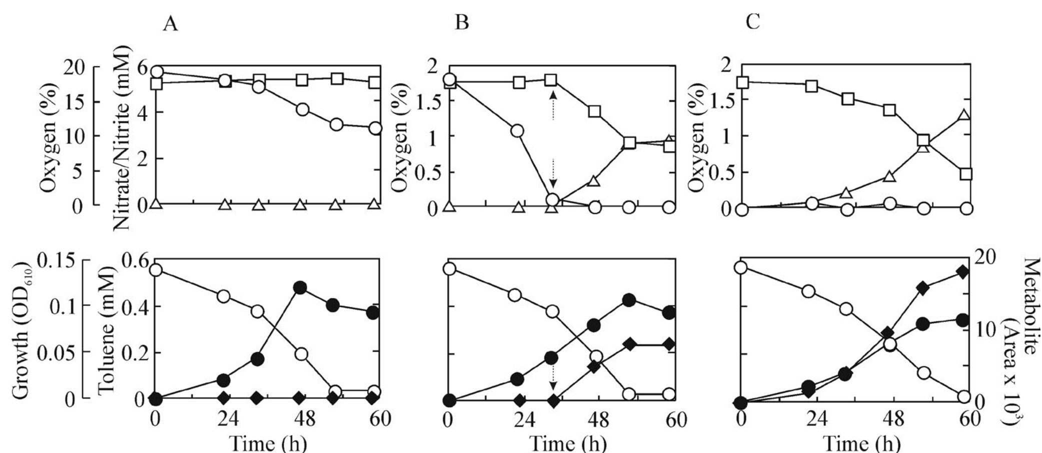


FIG. 2. Growth of *Thauera* sp. strain DNT-1 on toluene under aerobic (A), oxygen-limiting (B), and anaerobic (C) conditions, with 21, 2, and 0% oxygen in the gas phase of culture vessels, respectively. The amount of the metabolite was quantified by the area values of the peaks calculated by the recorder. The point of complete depletion of oxygen and the start of anaerobic degradation are indicated by arrows. Symbols in the top graphs: open circles, oxygen; open squares, nitrate; open triangles, nitrite. Symbols in the bottom graphs: open circles, toluene; closed circles, growth; closed diamonds, metabolite.

strain (see below). This strain has been deposited in the Japan Collection of Microorganisms (Saitama, Japan) under accession number JCM 12309.

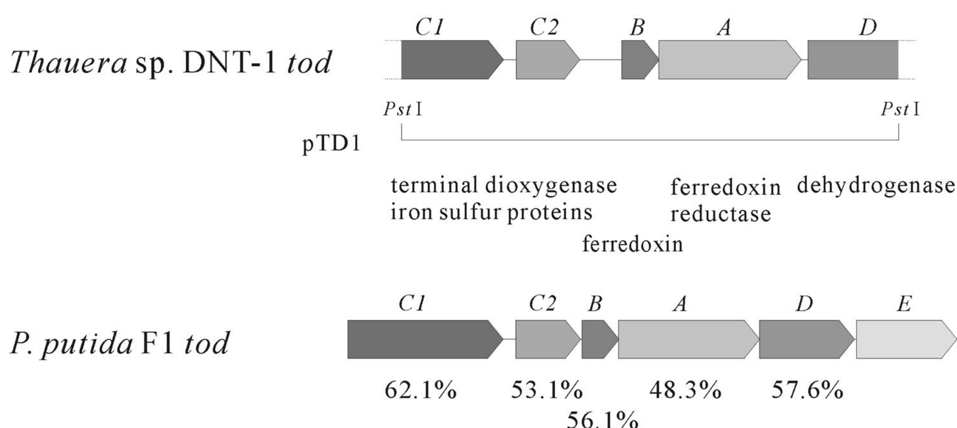
Toluene degradation under aerobic, oxygen-limiting, and anaerobic conditions. *Thauera* sp. strain DNT-1 was cultivated on toluene with different amounts of oxygen in sealed culture vessels (Fig. 2). When a sufficient amount of oxygen (21% O_2) was available, oxygen was consumed while toluene was degraded, and the nitrate concentration in the growth medium remained unchanged (Fig. 2A). Known intermediates of the five aerobic toluene degradation pathways, that is, benzoate, *p*-cresol, *m*-cresol, *o*-cresol, and 3-methylcatechol, were added to dense suspensions of strain DNT-1 cells grown aerobically on toluene. Among these compounds, only 3-methylcatechol was degraded instantly, and the cell suspension turned yellow, showing specific absorbance at 388 nm at pH 11.0 (data not shown).

Under anaerobic conditions (0% O_2), toluene degradation was accompanied by the reduction of nitrate and the accumulation of nitrite and an additional metabolite (Fig. 2C). The molecular weight of the metabolite was determined by LC/MS to be 206, the molecular weight of phenylitaconate. LC/MS analysis detected another compound in the culture fluid with a molecular weight of 208, the molecular weight of benzylsuccinate (data not shown). These compounds are known to accumulate in the culture fluids of *T. aromatica* K172 and *Azoarcus* sp. strain T1 (16, 32, 44), and phenylitaconyl CoA and benzylsuccinate are known to be intermediates in the anaerobic degradation of toluene by these strains (6, 7).

When 2% oxygen was initially present in the gas phase of the culture vessel, the organism continued to grow and degrade toluene while oxygen was depleted and nitrate reduction and nitrite accumulation started (Fig. 2B). When culture conditions changed from aerobic to anaerobic, the toluene metabolite also began to accumulate.

Cloning of putative genes encoding key enzymes of the aerobic and anaerobic toluene degradation pathway in *Thauera* sp. strain DNT-1. The 4.3-kb *Pst*I fragment (pTD1) detected by todC1p contains three complete and two partial open reading frames (ORFs) (Fig. 3A). Based on their similarity to genes identified in *P. putida* F1, they were named *todC1*, *todC2*, *todB*, *todA*, and *todD* and were predicted to encode the large and small subunits of the iron-sulfur protein of the terminal oxygenase, ferredoxin, and ferredoxin reductase of an aromatic compound-degrading multicomponent dioxygenase and the enzyme catalyzing the second step of the pathway, dihydrodiol dehydrogenase, respectively. The putative proteins TodC2, TodB, and TodA have calculated molecular masses of 21.7, 11.8, and 43.9 kDa, respectively, which are similar to the sizes of the toluene dioxygenase proteins from *P. putida* F1 deduced from the published nucleic acid sequence (52). The deduced amino acid sequences of *todC1C2BA* of strain DNT-1 show the highest similarity (59.0 to 87.6%) with the corresponding subunits of isopropylbenzene dioxygenase of *P. putida* RE204 (15), whereas the *todD* product is most similar (70.6%) to dihydrodiol dehydrogenase of the biphenyl degradation pathway of *Pseudomonas* sp. strain Cam-1 (31). The consensus amino acid sequences for the [2Fe-2S] cluster binding site, NAD^+- or FAD-binding site (30), and short-chain alcohol dehydrogenase superfamily (38) were found in the putative TodB (amino acids 43 to 45 and 63 to 66), TodA (amino acids 9 to 14 and 150 to 155), and TodD (Tyr-155 and Lys-159) proteins, respectively.

A 6.5-kb *Sac*I-*Bam*HI fragment composed of a segment spanning the 3.9-kb *Bam*HI (pTD18) fragment and the overlapping 6.0-kb *Sac*I (pTD21) fragment contained five ORFs (Fig. 3B). Based on their similarity to genes identified in *T. aromatica* K172, *T. aromatica* T1, and *Azoarcus* sp. strain T, the ORFs were named *bssD*, *bssC*, *bssA*, *bssB*, and *bssE*, and they are predicted to encode the benzylsuccinate synthase-activating enzyme, benzylsuccinate synthase γ-, α-, and β-subunits, and putative ATP or GTP binding protein of unknown function, respectively. The calculated molecular masses of the five putative proteins (42.5, 6.9, 97.6, 9.4, and 31.8 kDa, respec-



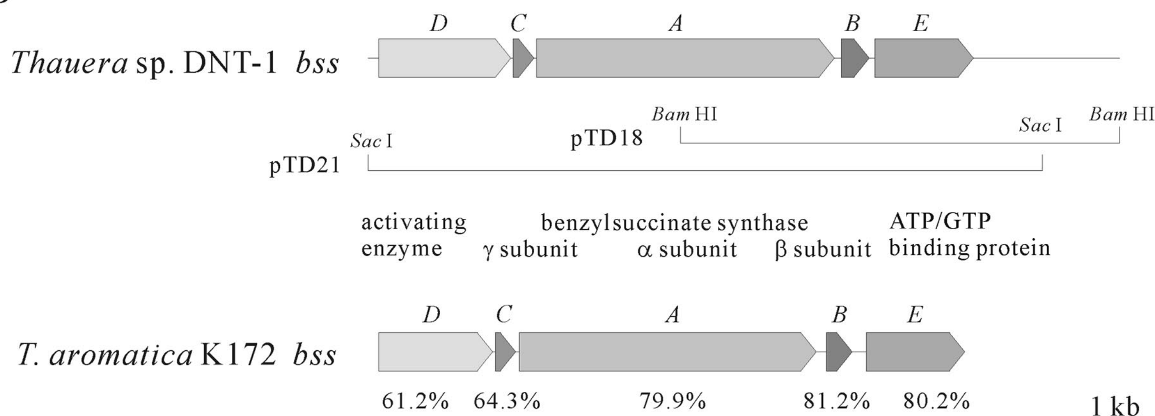


FIG. 3. Compositions and putative functions of *tod* (A) and *bss* (B) genes of *Thauera* sp. strain DNT-1 aligned with some of the most similar genes found in other aromatic compound-degrading bacteria. The percentages below the ORFs indicate the similarity of amino acid sequences to the corresponding ORF of strain DNT-1.

tively) and their deduced amino acid sequences are similar to those of the corresponding *bss* genes from *Thauera* and *Azoarcus* strains (61.2 to 81.2% similarity to those of *T. aromatica* K172, 88.4 to 98.0% with *T. aromatica* strain T1, and 78.2 to 97.6% with *Azoarcus* sp. strain T) (1, 13, 14, 28). The glycyl radical activation domain and two cysteine clusters of the ferredoxin consensus sequences, which were predicted to be amino acids 68 to 89, 106 to 116, and 140 to 150, respectively, are conserved in the putative BssD. The site of the glycyl radical and radical-accepting cysteine, which were predicted to be Gly-828 and Cys-492, respectively, were also conserved in putative BssA (1, 14, 28).

Expression of *tod* and *bss* genes. The sizes of the mRNA fragments that hybridized with the *todA* and *bssA* probes in the Northern blot analyses were each about 6 kb, which is longer than the currently sequenced regions of *todC1C2BAD* (4.3 kb) and *bssDCABE* (5.2 kb). Expression analyses of the *tod* genes of *P. putida* F1 revealed several transcripts of different lengths (9.5, 6.5, 2.5, and 1.6 kb) (50). Two different transcriptional organizations of the *bss* genes were found among *T. aromatica* K172 (24), *T. aromatica* T1 (13), and *Azoarcus* sp. strain T (1). The 6-kb transcripts of the *tod* and *bss* genes of *Thauera* sp. strain DNT-1 suggested polycistronic transcription of these genes, but further analysis is needed. Transcription of the *tod* genes was induced by toluene under aerobic conditions. Unexpectedly, however, the *bss* genes seemed to be transcribed under both aerobic and anaerobic conditions in the presence of toluene (Fig. 4). Further experiments using real-time QRT-PCR also suggested that transcription of the *tod* genes is induced from basal levels in aerobic cells grown on toluene, whereas transcription of the *bss* genes is induced by toluene regardless of the presence of oxygen (Fig. 5). Since a control experiment of reverse transcription without reverse transcriptase did not produce fluorescence within a reasonable number of cycles (data not shown), constitutive transcription of both sets of genes was suggested under every condition tested.

DISCUSSION

The newly isolated strain DNT-1 grew on toluene under both aerobic and anaerobic denitrifying conditions. DNT-1 degraded toluene by oxygen respiration first, and by nitrate

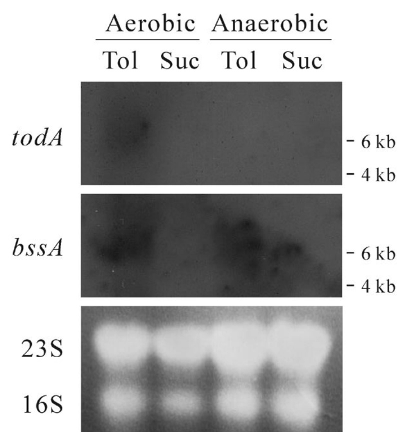


FIG. 4. Northern blot analyses of *todA* and *bssA* genes expressed in *Thauera* sp. strain DNT-1 grown under aerobic and anaerobic conditions with toluene (Tol) or succinate (Suc) as the sole carbon source. 16S and 23S rRNA bands visualized with ethidium bromide are shown under each lane to confirm that equal amounts of total RNA were loaded.

respiration under oxygen-limiting conditions with nitrate after the oxygen was consumed. We have concluded that strain DNT-1 degrades toluene by a dioxygenase pathway via 3-methylcatechol under aerobic conditions from the following results. (i) 3-Methylcatechol was instantly degraded in the reaction in the resting cell, and the reaction mixture transiently turned yellow, which was assumed to be derived from the *meta* fission product, 2-hydroxy-6-oxohepta-2,4-dienoate (5). (ii) Putative *todC1C2BADE* genes were cloned from the genomic DNA of strain DNT-1, and the deduced amino acid sequences were highly similar to the sequences of the *tod* genes of *P. putida* F1. (iii) The putative *tod* genes were transcribed in response to toluene and oxygen. The benzylsuccinate synthase pathway for the anaerobic degradation of toluene by this strain was demonstrated by the following results. (i) Phenylitaconate and benzylsuccinate, CoA thioesters of which are intermediates of the benzylsuccinate pathway, accumulated. (ii) A putative *bssDCABE* gene cluster was cloned from the chromosomal DNA of strain DNT-1, and the deduced amino acid sequences were revealed to be highly similar to the *bss* gene clusters from *T. aromatica* T1 (*tut* genes), *Azoarcus* sp. strain T (*bss* genes), and *T. aromatica* K172 (*bss* genes). (iii) The putative *bss* genes were transcribed in response to toluene.

The induction of transcription of the *bss* genes under aerobic conditions was unexpected. The putative product, benzylsuccinate synthase, is highly sensitive to oxygen, and the half-life of the activity in crude extract exposed to air is only 20 to 30 s (28). However, a trace amount of an inactive form of the oxygen-sensitive anaerobic ring reduction enzyme, benzoyl-CoA reductase, was detected in *T. aromatica* K172 cells grown aerobically in the presence of benzoate (23). Benzylsuccinate synthase may also be transcribed and weakly expressed under aerobic conditions in strain DNT-1 in preparation for an immediate response to environmental changes as shown in Fig. 2.

The background transcription of the *tod* and *bss* genes exhibited in QRT-PCR was another unexpected result. Expression of the *P. putida* F1 *tod* genes was reported to be induced by growth substrates and several other compounds (3, 11). The enzyme activity, protein, and mRNA of benzylsuccinate synthase are detected specifically in *T. aromatica* K172 cells grown with toluene (23, 28). Transcriptional analyses in *T. aromatica* T1 and *Azoarcus* sp. strain T, which were conducted by RT-PCR and Northern blotting, respectively, support these results (1, 13). Further investigation is needed to determine whether the basal level of transcription of the *tod* and *bss* genes in strain DNT-1 in the absence of toluene is due to a difference in the method used (real-time QRT-PCR) or differences in the strains.

In *P. putida* F1, the *tod* genes are supposedly regulated by a two-component signal transduction system encoded by the *todST* genes, which reside downstream of the *todXFC1C2BADEGIH* genes. In the presence of toluene, TodT binds to the promoter region of the *tod* genes (27). TodS is predicted to be a hybrid sensor kinase with two histidine kinase regions and an oxygen-sensing region. The role of TodS as a sensor for detecting inducers has not been clearly demonstrated. Its function in sensing aromatic substrates is suggested by the observations that point mutations in this gene result in *P. putida* F1 mutants with broader substrate specificities (12), and inactivation of the gene results in the loss of the ability to sense toluene by chemotaxis (36). However, there is currently no evidence indicating that TodS is involved in oxygen sensing. The regulation system of the *tod* genes in strain

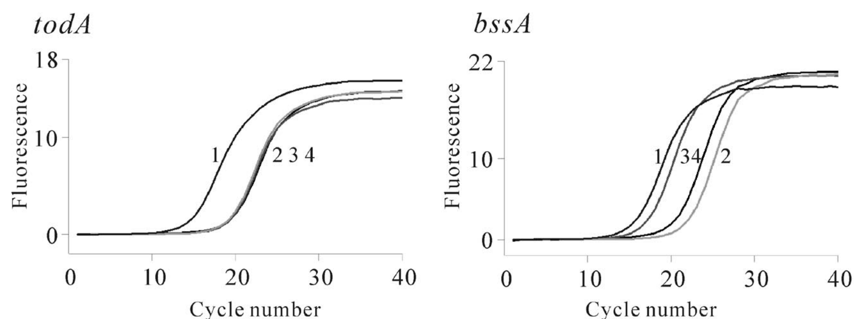


FIG. 5. Kinetic PCR curves of real-time QRT-PCR targeting *todA* and *bssA* genes. Relative fluorescence output is plotted versus PCR cycle number. Kinetic curves for cells grown aerobically in toluene (line 1) or succinate (line 2) and for cells grown anaerobically in toluene (line 3) or succinate (line 4) are shown.

DNT-1, which are controlled by the presence of both toluene and oxygen, is now under investigation.

Aerobic and anaerobic toluene degradation pathways and the expression of the putative initial enzymes of each pathway were studied in a newly isolated toluene degrader, *Thauera* sp. strain DNT-1. Investigation of the regulation mechanisms of the *bss* and *tod* genes in this strain is particularly interesting when a possible relationship with the regulation of respiratory pathways (Fig. 2) is considered. A comprehensive understanding of these regulatory systems is also valuable for practical purposes, such as the application of these bacteria to in situ bioremediation processes, in which oxygen availability varies at different polluted sites and at different times.

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