Applied and Environmental Microbiology, Dec. 2008, p. 7313–7320 Vol. 74, No. 23
0099-2240/08/$08.00+0 doi:10.1128/AEM.01695-08
Copyright © 2008, American Society for Microbiology. All Rights Reserved.

Influence of Soil Components on the Biodegradation of Benzene, Toluene, Ethylbenzene, and *o*-, *m*-, and *p*-Xylenes by the Newly Isolated Bacterium *Pseudoxanthomonas spadix* BD-a59[▽]

Jeong Myeong Kim,[1]† Ngoc Thuan Le,[2]† Bok Sil Chung,[1] Jin Ho Park,[2] Jin-Woo Bae,[3] Eugene L. Madsen,[4] and Che Ok Jeon[1]*

Department of Life Science, Chung-Ang University, HeukSeok-Dong, Seoul 156-756, Republic of Korea[1]; Environmental Biotechnology National Core Research Center, Gyeongsang National University, Jinju 660-701, Republic of Korea[2]; Biological Resource Center, KRIBB, Daejeon 305-806, Republic of Korea[3]; and Department of Microbiology, Cornell University, Ithaca, New York 14853-8101[4]

Received 22 July 2008/Accepted 30 September 2008

A bacterium designated strain BD-a59, able to degrade all six benzene, toluene, ethylbenzene, and *o*-, *m*-, and *p*-xylene (BTEX) compounds, was isolated by plating gasoline-contaminated sediment from a gasoline station in Geoje, Republic of Korea, without enrichment, on minimal salts basal (MSB) agar containing 0.01% yeast extract, with BTEX as the sole carbon and energy source. Taxonomic analyses showed that the isolate belonged to *Pseudoxanthomonas spadix*, and until now, the genus *Pseudoxanthomonas* has not included any known BTEX degraders. The BTEX biodegradation rate was very low in MSB broth, but adding a small amount of yeast extract greatly enhanced the biodegradation. Interestingly, degradation occurred very quickly in slurry systems amended with sterile soil solids but not with aqueous soil extract. Moreover, if soil was combusted first to remove organic matter, the enhancement effect on BTEX biodegradation was lost, indicating that some components of insoluble organic compounds are nutritionally beneficial for BTEX degradation. Reverse transcriptase PCR-based analysis of field-fixed mRNA revealed expression of the *tmoA* gene, whose sequence was closely related to that carried by strain BD-a59. This study suggests that strain BD-a59 has the potential to assist in BTEX biodegradation at contaminated sites.

A considerable amount of gasoline is released into soil and sediments due to leakage from underground storage tanks, accidental spills, or improper waste disposal practices. The benzene, toluene, ethylbenzene, and *o*-, *m*-, and *p*-xylene (BTEX) compound is one of the most common groundwater and soil contaminants (7). Because of their high solubility in water relative to that of other petroleum hydrocarbons, these compounds account for as much as 90% of the gasoline components found in the water-soluble fraction, and groundwater can transport them from tens to hundreds of meters down gradient of the contamination source (5, 28). BTEX compounds are toxic to humans and are confirmed or suspected carcinogens (10); thus, the United States EPA classifies them as environmental priority pollutants, making their removal from polluted environments critical (10). Bacteria that degrade BTEX compounds under aerobic conditions are widely distributed, and researchers have isolated many of these strains by enrichment broth culturing with BTEX compounds as the sole carbon and energy sources, under aerobic and anaerobic conditions (16, 29, 36). These results have led to extensive studies of the metabolism and genetics of BTEX degraders, mostly *Pseudomonas* species or closely related *Ralstonia* and *Burkholderia* species, because these kinds of bacteria grow well on minimal medium or under laboratory culture conditions (1, 21, 25, 37).

Except for *Pseudomonas* or closely related species, only a few isolated microorganisms are known to degrade BTEX compounds, probably because isolation techniques have focused largely on laboratory enrichment procedures using BTEX as the sole carbon and energy source. The potentially vast array of bacteria that cannot grow or enrich under these conditions led us to ask whether as-yet-undiscovered BTEX degraders may require other growth conditions. In this study, we isolated a *Pseudoxanthomonas* species (strain BD-a59) without enrichment by direct plating of gasoline-contaminated soil on minimal salts basal (MSB) agar containing 0.01% (wt/vol) yeast extract (YE), with BTEX as the sole carbon source. The isolate was able to degrade all six BTEX compounds, and we evaluated the influence of soil components on BTEX biodegradation by strain BD-a59.

MATERIALS AND METHODS

Soil samples and chemicals. The gasoline-contaminated soil sample used to isolate BTEX-degrading bacteria was obtained from a gasoline station in Geoje, Republic of Korea (34°85′N, 128°65′E), in March 2006. After samples were collected in sterile plastic tubes, one soil sample for the isolation of BTEX-degrading bacteria was stored in an icebox for the time of transport. Another soil sample for the isolation of mRNA was rapidly frozen in the field by placing 20 g of soil into a screw-cap, sterile plastic tube and immersing it in ethanol mixed with dry ice; this sample was later transferred to a −80°C freezer. The nutritional impact of the contaminated soil and an uncontaminated soil sample (obtained from a playground on the Gyeongsang National University campus) on BTEX metabolism by strain BD-a59 was examined. The contaminated and uncontaminated soil samples were analyzed for soil particle size, organic matter content, and soil pH. The distribution of the soil particles was determined by particle

* Corresponding author. Mailing address: Department of Life Science, Chung-Ang University, 221 HeukSeok-Dong, Seoul 156-756, Republic of Korea. Phone: 82-2-820-5864. Fax: 82-2-821-8132. E-mail: cojeon@cau.ac.kr.
† J. M. Kim and N. T. Le contributed equally to these studies.
▽ Published ahead of print on 3 October 2008.

TABLE 1. Primer sets used in this study

Primer set	Target gene	Sequence (5′–3′)	Expected size (bp)	Source or reference
TBMD-F TBMD-R	Subfamily 1 of α subunit of hydroxylase component of multicomponent monooxygenases	GCCTGACCATGGATGCSTACTGG CGCCAGAACCACTTGTCRRTCCA	640	14
TMOA-F TMOA-R	Subfamily 1 of α subunit of hydroxylase component of multicomponent monooxygenases	CGAAACCGGCTTYACCAAYATG ACCGGGATATTTYTCTTCSAGCCA	505	14
TOL-F TOL-R	Subfamily 5 of hydroxylase component of two-component side chain monooxygenases	TGAGGCTGAAACTTTACGTAGA CTCACCTGGAGTTGCGTAC	475	6
XYLA-F XYLA-R	Electron transfer component of two-component side chain monooxygenases	CCAGGTGGAATTTTCAGTGGTTGG AATTAACTCGAAGCGCCCACCCCA	291	14
TODC1-F TODC1-R	Subfamilies D.1.B, D.1.C, D.2.A, D.2.B, and D.2.C of α subunit of type D iron-sulfur multicomponent aromatic dioxygenases	CAGTGCCGCCAYCGTGGYATG GCCACTTCCATGYCCRCCCCA	510	14
RIESKE-F RIESKE-R	Rieske nonheme iron dioxygenases	TGYCGBCAYCGBGGSAWG CCAGCCGTGRTARSTGCA	78	14
XYLE-F XYLE-R	Subfamily 1.2.A of catechol extradiol dioxygenases	CCGCCGACCTGATCWSCATG TCAGGTCAKCACGGTCAKGA	934	14
NahAc-F NahAc-R	Naphthalene dioxygenases	TATCACGGCTGGKSYTTCGGCT CTGGCWWTTYCTCACYCATGA	480	33
16S F1 16S R13	Eubacterial 16S rRNA genes	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT	1,542	22
M13 reverse	pCR2.1 vector	CAGGAAACAGCTATGAC	Sequencing	Invitrogen
T7 promoter	pCR2.1 vector	TAATACGACTCACTATAGGG	Sequencing	Invitrogen

sedimentation, using the hydrometer method (4), and led to classifying both soils as sandy loams. The organic matter content in the soil samples was determined according to the ASTM International standard method D2974 (2), based on mass loss following dry sample ignition at 500°C. Soil pH analysis (6.4 to 6.6) was performed using potentiometry (electrode model 25; Accumet) according to ASTM International standard method D4972 (3). Total petroleum hydrocarbons were quantified using a previously described method (11). Chemicals for MSB medium preparation (30) were purchased from Sigma-Aldrich, and all other chemicals used in this study were of analytical grade. Difco Noble agar (BD) was used to screen for BTEX-degrading bacteria. Sodium pyruvate (10% [wt/vol]) solution was filter sterilized prior to the addition to media.

Growth media and conditions for isolation and cultivation. Four sets of 20 bacterial strains (80 total) were isolated from the contaminated soil. For two sets, serial dilutions (0.9% [wt/vol] saline) were directly plated onto two different media (MSB agar with and without 0.01% [wt/vol] YE). To obtain the other two sets of 20 isolates, we used BTEX broth enrichments (one set with and the other set without YE) inoculated with the contaminated soil. A 1,000-mg/liter BTEX mixture (benzene, toluene, ethylbenzene, and *o*-, *m*-, and *p*-xylene [1:1:1:1:1:1]) was the major source of carbon and energy. The BTEX mixture was replenished to the initial concentration every 2 to 3 days to compensate for the loss of BTEX due to volatilization or use by microorganism; the cultures were shaken at 25°C and 150 rpm until turbidity was noticeable (approximately 10 to 15 days). After three serial transfers into fresh enrichment medium, the growing cultures were plated on MSB agar plus 0.01% YE, as appropriate.

For all four isolation procedures, after samples were spread on MSB agar plates, the plates were incubated for 10 days at 25°C in a BTEX atmosphere, which was produced by a mixture of BTEX compounds dissolved in vacuum pump oil within a reservoir placed inside a sealed plastic box (15). Colonies exhibiting strong growth were picked and streaked on fresh R2A agar to isolate single pure colonies. The ability of the isolates to grow by biodegrading BTEX was initially evaluated by comparing their colony sizes on MSB agar containing 0.01% YE, with and without BTEX compounds.

Identification of isolates and phylogenetic analysis. Colonies exhibiting good growth on MSB agar with BTEX compounds were resuspended in 100 μl of 5% (wt/vol) Chelex-100 solution (Bio-Rad) and boiled for 10 min to prepare crude genomic DNA lysates. PCR amplification of 16S rRNA genes from the crude lysates was performed using the F1 and R13 primer set (Table 1) as described previously (24), and the PCR amplicons were double digested with HaeIII and HhaI. Restriction fragment length polymorphism (RFLP) patterns were analyzed on 2.5% (wt/vol) MetaPhore agarose (BioWhittaker) gels alongside a 100-bp ladder (Bioneer, Korea), and colonies were grouped according to their RFLP patterns, as described previously (17). Representative PCR products containing unique RFLP fragment patterns were ligated into the pCR2.1 vector (Invitrogen) according to the protocol recommended by the manufacturer and then sequenced using the T7 promoter and M13 reverse primers (Table 1). The resulting 16S rRNA gene sequences were compared to GenBank entries via BLASTN searches, and the search results were used as a guide to classify the isolates. To construct a phylogenetic tree for strain BD-a59, 16S rRNA gene sequences were aligned with corresponding GenBank sequences from representative members of selected genera using Clustal W software, and a phylogenic tree was constructed using the algorithm of the Kimura two-parameter model for the neighbor-joining method of PHYLIP software, version 3.6 (PHYLIP, Seattle, WA). DNA-DNA hybridization was carried out to evaluate the genomic DNA

relatedness between strain BD-a59 and *Pseudoxanthomonas spadix* IMMIB AFH-5[T], using a modified fluorometric microplate method (12, 23).

BTEX transformations in liquid medium. The biodegradation ability of strain BD-a59 was evaluated with individual BTEX compounds (benzene, toluene, ethylbenzene, or *o*-, *m*-, and *p*-xylenes) and a mixture in 160-ml serum bottles containing 10 ml of MSB medium and YE (if appropriate) under aerobic conditions in triplicate. Strain BD-a59 cells were cultured in R2A broth at 25°C for 24 h, harvested by centrifugation (8,000 $\times$ *g*, 5 min), washed with MSB broth, and repelleted. The cells were then resuspended in fresh MSB broth to a density of approximately 10^8 cells/ml, and 50 μl of the resuspended cells was used to seed serum bottles. The serum bottles were sealed with Teflon-coated gray butyl rubber septa and aluminum crimp caps, and the BTEX compounds were injected into the bottles with a microsyringe, individually (50 mg/liter) or as a mixture (30 mg/liter each). Uninoculated serum bottles (no cells; MSB medium and YE only) were prepared as negative controls. The serum bottles were incubated at 25°C and 150 rpm; periodically, three bottles were sacrificed at each sampling time, and the contents were extracted with 5 ml of methylene chloride and analyzed for BTEX concentrations, using gas chromatography, as described below.

BTEX transformations in soil slurry systems. The effects of soil solids on BTEX biodegradation of strain BD-a59 were investigated using a previously described procedure (9) with some modifications. Three grams (wet weight) of the contaminated soil was transferred into sterile 160-ml serum bottles containing 10 ml of MSB; then, the serum bottles were autoclaved for 15 min at 121°C, and the serum bottles were sealed with Teflon-coated gray butyl rubber septa and aluminum crimp caps. Strain BD-a59 cells (prepared as described above for liquid medium transformation) were inoculated into the serum bottles, and a BTEX substrate mixture (30 mg/liter of each component) was injected into the bottles with a microsyringe. Uninoculated serum bottles were prepared as negative controls. The serum bottles were incubated at 25°C and 150 rpm, after which the BTEX analysis protocols described above were followed. BTEX compounds were analyzed as described below.

BTEX transformations in soil extract and soil sediment. The effects of unknown soil components on BTEX biodegradation by strain BD-a59 inoculated into serum bottles, as described above, were investigated using contaminated and uncontaminated soil, as follows. Thirty grams of soil was suspended in 100 ml of MSB broth and agitated overnight. The soil slurries were centrifuged at 8,000 $\times$ *g* for 20 min; 10 ml of the supernatant was dispensed into 160-ml serum bottles, and 3 g of the wet sedimented pellet was resuspended in serum bottles containing 10 ml of MSB broth. The serum bottles were sealed with Teflon-coated gray butyl rubber septa and aluminum crimp caps and autoclaved for 15 min at 121°C.

For the biodegradation test using combusted soil, the contaminated soil was combusted at 550°C for 3 h to remove organic matter from the soil sediment, and 2 g of the combusted soil was resuspended in serum bottles containing 10 ml of MSB broth, which were sealed with Teflon-coated gray butyl rubber septa and aluminum crimp caps. Uncombusted contaminated soil was used as a positive control. The serum bottles were autoclaved for 15 min at 121°C. BTEX injection, cell inoculation, serum bottle incubation, and the BTEX analysis were performed as described above.

BTEX analysis. At each sampling time, three serum bottles were sacrificed, and BTEX compounds in serum bottles were extracted with 5 ml of methylene chloride for at least 1 h by inversion. The lower methylene chloride layer containing the BTEX compounds (2 ml) was removed, centrifuged at 3,000 $\times$ *g* for 5 min, and subjected to BTEX analyses. Prior to extraction, an internal naphthalene standard was added to the methylene chloride to a final concentration of 50 μg/ml. Gas chromatography analyses were performed with a model 6890N gas chromatograph (Agilent Technologies) with an HP-5 capillary column (30-m length, 0.32-mm inner diameter, 0.25-μm film thickness [J & W Scientific]) coupled to a flame ionization detector. The gas chromatography oven was programmed to increase from 60°C (held for 1 min) to 220°C at 10°C/min, after which 220°C was held for 3 min. The gas flow to the detector contained H_2 (40 ml/min) and synthetic air (450 ml/min), the detector temperature was 300°C, the injection port temperature was 250°C, and the 1-μl samples were loaded with an auto sampler with a split mode (5:1). The profiles of *m*-xylene and *p*-xylene mirrored each other, because they had the same retention time on the gas chromatography analysis chromatogram (calculated by dividing by 2).

PCR amplification of BTEX catabolic genes from genomic DNA of strain BD-a59 and mRNA of the contaminated soil. All PCR primers used in this study are listed in Table 1. PCR amplification was carried out in a 50-μl reaction mixture containing purified DNA from strain BD-a59. Other PCR amplifications and analyses were carried out according to the procedures that have been described previously (6, 14). Primer sets F1/R13, TOL-F/R, and TODC1-F/R for strain BD-a59, *Pseudomonas putida* mt-2, and *Ralstonia pickettii* PKO1 were used as positive controls, respectively (6). The PCR products were ligated into the pCR2.1 vector (Invitrogen) and then sequenced using an ABI model 3700 instrument at Genotech (Korea).

Reverse transcriptase PCR (RT-PCR) was carried out to assess whether strain BD-a59 was involved in the metabolism of BTEX at the contaminated site. Total RNA was prepared from the contaminated soil sample using an RNA PowerSoil total RNA isolation kit (MO BIO) as recommended by the manufacturer, and other detailed methods for the RNA extraction have been described elsewhere (18). The RNA extract was treated to remove any genomic DNA contaminants by incubation with 1 U of RNase-free DNase I (Promega) and 1 U of RNasin (Promega) in 40 mM Tris-HCl (pH 7.9) containing 10 mM NaCl, 10 mM $CaCl_2$, and 6 mM $MgSO_4$ for 30 min at 37°C. The RNA preparation was cleaned by passage through an RNase mini-column (Qiagen) prior to use in RT-PCR. The RT-PCR was carried out by using SuperScript II RT (Invitrogen Life Technologies, Carlsbad, CA) and the TMOA-F/R primer set. The RT-PCR product was cloned into the pCR2.1 vector, and the nucleotide sequences of four clones were determined using the T7 promoter and M13 reverse primers (Table 1).

The resulting oxygenase gene sequences were subjected to GenBank for BLASTX searches. A neighbor-joining tree showing the phylogenetic relationships among the oxygenase genes was constructed based on sequence alignment using PROTDIST and neighbor modules of PHYLIP version 3.62 software (http://evolution.genetics.washington.edu/) with the Jones-Taylor-Thornton matrix (version 3.6a; PHYLIP, Seattle, WA).

RESULTS AND DISCUSSION

Isolation and identification of the BD-a59 BTEX degrader from a gasoline-contaminated site. Bacterial strains were isolated from a gasoline-contaminated soil sample containing ~4,500 mg/kg total petroleum hydrocarbons, using four different procedures. The procedures collectively yielded 80 isolates (20 from each procedure) whose growth on MSB agar was clearly enhanced by the presence of BTEX vapor. The isolates' 16S rRNA genes were PCR amplified, double digested with a mixture of HaeIII and HhaI, and sorted by their RFLP patterns. Isolates with unique RFLP fragment patterns were sequenced and analyzed using a BLASTN search for their phylogenetic identification. The direct plating isolation procedure employing the YE-free MSB medium yielded culturable BTEX-degrading bacteria that were mostly *Pseudomonas*-related species (18/20), with some *Acinetobacter*-related species (2/20). The two broth enrichment procedures (1,000 mg/liter BTEX [plus 0.01% YE]) yielded isolates exclusively in the genus *Pseudomonas*. This finding confirmed the fact that *Pseudomonas* species respond well to BTEX in laboratory enrichment culture. In contrast, though the direct plating procedure (no enrichment) employing MSB plus 0.01% YE yielded largely *Pseudomonas* species (14/20), this procedure also revealed several *Acinetobacter*-related (2/20) and *Pseudoxanthomonas*-related species (3/20). Many other studies have reported BTEX-degrading bacteria belonging to the genera *Pseudomonas* and *Acinetobacter*, but to our knowledge, no one has reported a BTEX-degrading *Pseudoxanthomonas* species. For this reason, we characterized the BTEX biodegradation potential of a *Pseudoxanthomonas*-related isolate, which was designated strain BD-a59 (DSM 19372 and KCTC 22081).

Bacteria previously identified as BTEX degraders in soil environments include species from the genera *Pseudomonas*, *Burkholderia*, *Sphingomonas*, *Thauera*, *Dechloromonas*, *Rhodococcus*, and *Acinetobacter* (8, 19, 20, 26, 29, 38). Most of these bacteria were isolated by enrichment using minimal medium or by spreading on minimal agar medium (without added YE) in the presence of BTEX vapor as the sole carbon and energy source. However, such traditional approaches for isolating BTEX degraders are successful only for bacteria that compete

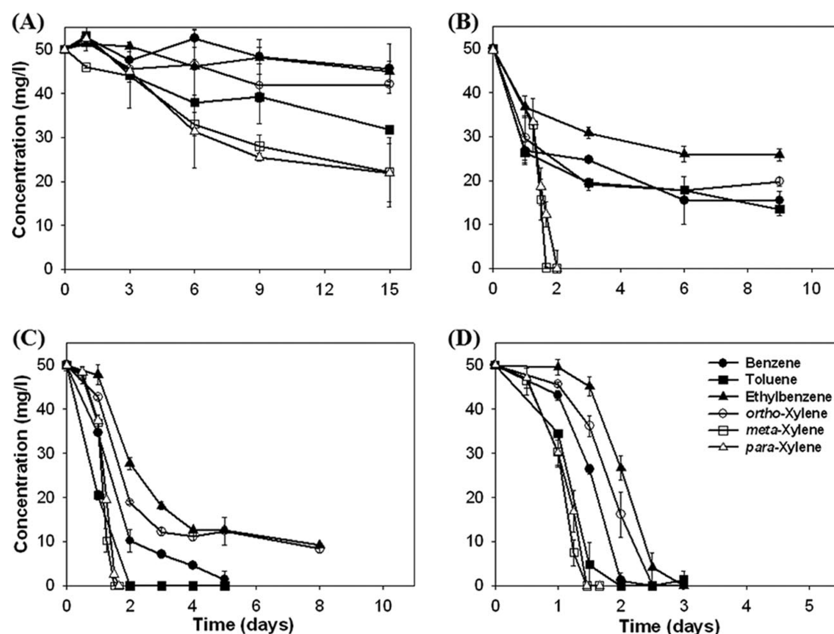

FIG. 1. Effects of YE on degradation of individual BTEX compounds (initial concentration of each BTEX compound, 50 mg/liter) by strain BD-a59 in mineral salt medium without YE (A), with 10 mg/liter of YE (B), with 20 mg/liter of YE (C), and with 50 mg/liter of YE (D). Concentrations of BTEX compounds were determined by gas chromatography. The uninoculated sterile control showed no biodegradation activity. The averages of triplicate experiments ± standard errors of the means, indicated by error bars, are shown.

well with others in the presence of high concentrations of BTEX components in laboratory media. Countless other BTEX-degrading bacteria may be present in the soil or sediment but may not compete well in aqueous slurries or perhaps cannot grow in minimal medium; thus, this alternative pool of BTEX-degrading populations is likely to be overlooked. Our study reinforced this concept.

Strain BD-a59 was a yellow-pigmented, gram-negative, nonmotile, oxidase- and catalase-positive rod bacterium that was 1.5 to 2.0 μm long and 0.7 to 0.9 μm wide. Its growth was observed in the range of 15 to 35°C (optimum, 25 to 30°C) and between pH 6.0 and 9.0 (optimum, pH 7.5 to 8.0). The G + C content of its genomic DNA was about 67.7 mol%. Strain BD-a59 grew well on R2A agar, but it did not grow on MSB agar containing 0.3% (wt/vol) pyruvate or on LB agar. However, the strain was able to grow on MSB agar containing 0.3% (wt/vol) pyruvate when it was also supplemented with 0.01% YE.

Phylogenetic analysis based on the 16S rRNA gene sequence (GenBank accession no. EF575561) indicated that strain BD-a59 is a member of the gammaproteobacterial genus *Pseudoxanthomonas*, with a 99.4% sequence similarity to *P. spadix* IMMIB AFH-5^T (data not shown). It exhibited >90% DNA-DNA relatedness to *P. spadix* IMMIB AFH-5^T, which is clearly above the 70% threshold generally accepted as the same species (27). Based on these results, as well as chemotaxonomic and molecular characteristics, strain BD-a59 was assigned as a member of the *P. spadix* species. *P. spadix* IMMIB AFH-5^T has also been isolated from oil-contaminated soil (35), but it did not exhibit BTEX degradation ability, and degenerate oxygenase PCR primers did not amplify PCR products from this strain (data not shown).

BTEX degradation tests with MSB broth. The BTEX degradation abilities of strain BD-a59 were evaluated using 50 mg/liter of each individual substrate or a BTEX mixture containing 30 mg/liter of each component in MSB broth. In uninoculated-control experiments, BTEX loss during culture was negligible (data not shown). Figure 1 shows the individual BTEX concentration profiles with respect to incubation time. When the MSB did not contain YE, BTEX degradation was very slow, especially for benzene, ethylbenzene, and *o*-xylene (Fig. 1A). As the amount of YE added was increased, BTEX degradation improved markedly; in fact, the BTEX was completely consumed within 3 days in cultures containing 50 mg/liter YE (Fig. 1D). To further define the degradation relationships among the six BTEX compounds, we determined degradation profiles for each compound in the mixture (1:1:1:1:1:1 [Fig. 2]). The BTEX degradation in the mixture was improved as the amount of YE increased, which was the same as those of individual BTEX compounds. The degradation rate of the BTEX mixture increased with increasing amounts of YE; at 150 mg/liter YE, the BTEX mixture was completely degraded in 3 days (Fig. 2D).

Because YE can also serve as a carbon source for microorganisms, we hypothesized that initially, YE would allow cell numbers to increase in the medium, possibly to overcome a density-dependent inhibition of growth by the BTEX compounds. Alternatively, the YE might contain inducers that were necessary for efficient expression of the BTEX degradation genes, as previously suggested by Hatzinger et al. (13) in their study of methyl *tert*-butyl ether degradation. However, both of these reasons were excluded, because strain BD-a59 grew well in MSB containing YE using pyruvate, but it did not grow in MSB without YE. Thus, it appears that YE contains

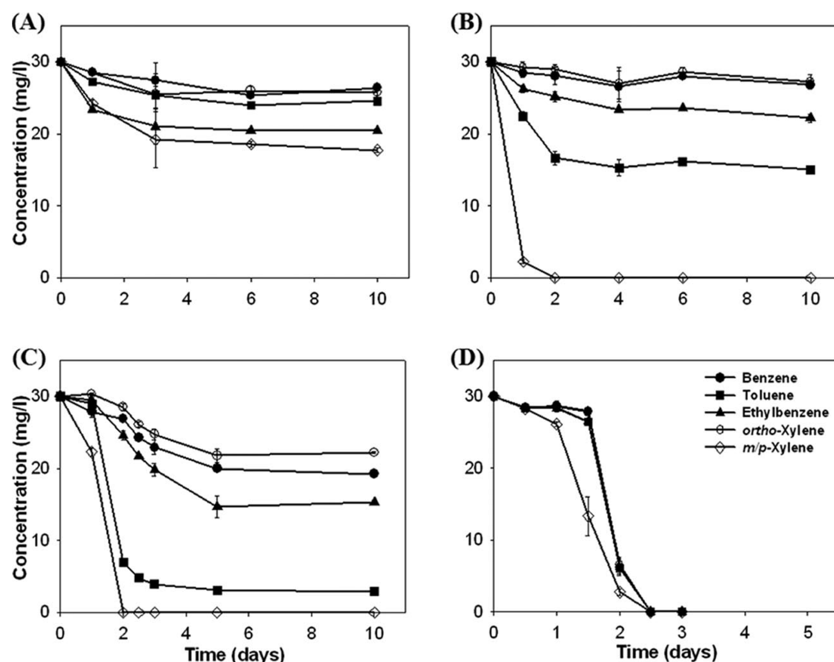

FIG. 2. Effects of YE on biodegradation of a BTEX mixture (30 mg/liter for each component) by strain BD-a59 in mineral salt medium without YE (A), with 20 mg/liter of YE (B), with 50 mg/liter of YE (C), and with 150 mg/liter of YE (D). Concentrations of BTEX compounds were determined by gas chromatography. The uninoculated sterile control showed no biodegradation activity. The averages of triplicate experiments ± standard errors of the means, indicated by error bars, are shown.

growth factors such as vitamins that are essential for the growth of strain BD-a59 but that are not found in MSB.

Enhanced biotransformation of BTEX in a soil slurry system. Since the above-described results had demonstrated that MSB was inappropriate for strain BD-a59 isolation and BTEX biodegradation (the strain could not grow on MSB, even with pyruvate, if there was no YE), BTEX compound biotransformation tests were conducted in a soil slurry system to determine whether soil conditions might be appropriate for strain BD-a59 growth or if the soil contained unknown factors and inducers that could support BD-a59 in the bioremediation processes. When strain BD-a59 cells were inoculated into a sterile slurry of contaminated soil containing a mixture of BTEX, strain BD-a59 completely degraded all BTEX compounds within 2 days (data not shown), demonstrating that some of the soil components clearly enhanced BTEX biodegradation. BTEX persisted in the uninoculated negative control.

BTEX transformations in soil extract and soil sediment. Soil and sediment systems are complex; they may contain many inorganic or organic components and essential trace elements able to support the growth of microorganisms. Therefore, based on the results from degradation tests with the soil slurry systems, characteristics of soil components having a potentially positive effect on the biodegradation of BTEX compounds were investigated. For the tests, two kinds of soil, contaminated and uncontaminated, were used, both of which were sandy loam types with a slightly acidic pH (6.4 to 6.6). The contaminated soil contained more organic matter (2.7%) than the uncontaminated soil (1.3%). The two soil samples were fractionated into aqueous soil extracts and soil solids; Fig. 3 shows the BTEX mixture degradation by strain BD-a59 exposed to each fraction. BTEX biodegradation occurred very slowly in the aqueous extracts of the contaminated and uncontaminated soil (Fig. 3A and C), similar to what we had observed in the MSB broth without YE. In contrast, strain BD-a59 completely depleted the BTEX from the sediment slurry systems from contaminated and uncontaminated soil within 2 days and 6 days, respectively (Fig. 3B and D). The contaminated sediment, with its higher organic matter content, caused a higher BTEX biodegradation rate than the uncontaminated sediment, most likely because the contaminated soil contained more growth factors. In support of this idea, soil from which the organic matter was removed by burning no longer exhibited enhanced BTEX biodegradation by BD-a59 (data not shown), indicating that nutrient components essential for the growth of strain BD-a59 might be water-insoluble organic matter attached to soil particles and that this stimulating organic matter (present in both contaminated and uncontaminated soil) was analogous to YE in stimulating BTEX metabolism. Importantly, these results suggested that strain BD-a59 may not need exogenous YE in bioremediation applications at contaminated sites. Venkateswarlu and Sethunathan (32) reported enhanced biodegradation of carbonfuran by *Pseudomonas cepacia* and *Nocardia* sp. in the presence of growth factors from YE and aqueous soil extract. In contrast, BTEX biodegradation by strain BD-a59 was enhanced by the soil solids, not by an aqueous soil extract.

As discussed by Wolf and Skipper (34), there is no artifact-free procedure for preparing sterile soil because chemical changes created by autoclaving and/or gamma irradiation and/or adding inhibitors of microbial activity are absent from

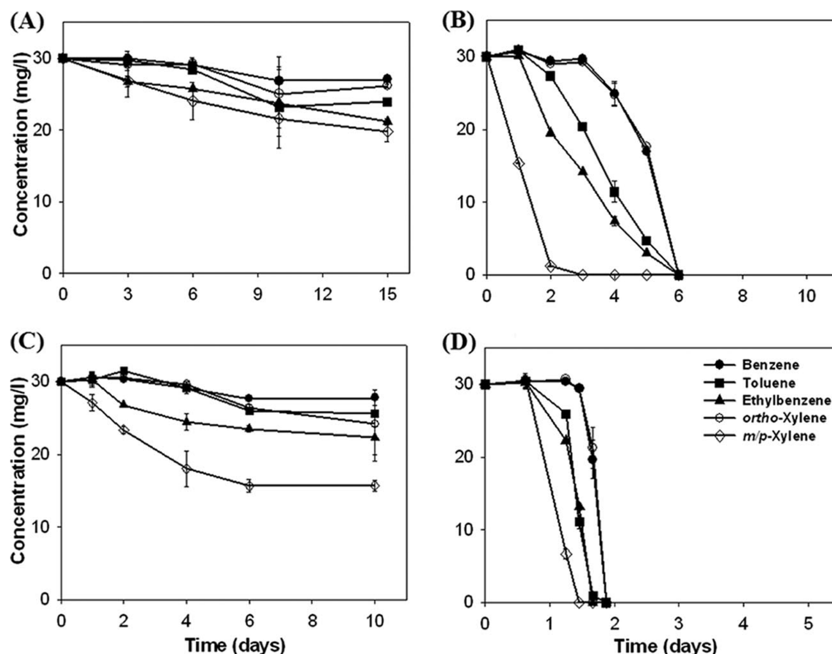

FIG. 3. Effects of various sterile soil and sediment amendments on biodegradation of a BTEX mixture (30 mg/liter, each component) by strain BD-a59 in serum bottles. Treatments consisted of uncontaminated aqueous soil extract (A), 30% ([wt/vol] wet weight base) uncontaminated soil in MSB broth (B), contaminated aqueous soil extract (C), and 30% ([wt/vol] wet weight base) contaminated soil sediment in MSB broth (D). Concentrations of BTEX compounds were determined by gas chromatography. The uninoculated sterile control showed no biodegradation activity. The averages of triplicate experiments ± standard errors of the means, indicated by error bars, are shown.

the live treatments. In experiments reported here, our goal was to assess the influence of chemical/physical components of soil upon biodegradation carried out by strain BD-a59. To achieve complete sterilization, it is recommended that soil be autoclaved for 1 h on day 1 and then, after 2 days, that the soil be autoclaved again for 1 h on day 3 (34). We chose to autoclave the soil once for 15 min because, though some heat-resistant microorganisms (e.g., spores) may have survived, autoclaving was effective at eliminating BTEX metabolism in the absence of strain BD-a59 (Fig. 3) but presumably minimized the drastic changes in the chemical composition of the soil that can result from consecutive 1-h autoclaving periods.

PCR amplification of BTEX-catabolic genes from strain BD-a59 and soil mRNA. To date, only a limited set of isolated microorganisms, including *Ralstonia pickettii* PKO1 and *Dechloromonas* sp. strain RCB, have been reported to degrade all six BTEX compounds (8, 26). Strain BD-a59 also has the ability to degrade all BTEX compounds, which adds significantly to this small set. To assess strain BD-a59's complement of oxygenase genes related to BTEX degradation, eight degenerate primer sets were applied to BD-a59 DNA for PCR amplification (Table 1). PCR amplicons were produced from the primer sets NahAc-F/R, TBMD-F/R, TMOA-F/R, and TODC1-F/R and were sequenced (Fig. 4). The gene sequence of the PCR amplicon produced from the NahAc-F/R set was not related to aromatic compound degradation (amino acid identity to phage-related protein of *P. aeruginosa* PA7, 58%), which meant that this was a nonspecific PCR amplicon. Analysis of other PCR amplicons showed that strain BD-a59 contained one dioxygenase and two different monoxygenase genes (Fig. 5) whose expected hosts were phylogenetically very divergent from each other. One monoxygenase fragment, amplified from the TBMD-F/R primer set, was approximately 640 bp long (GenBank accession no. EU734589) and was quite similar to the *R. pickettii* 12J methane/phenol/toluene hydroxylase gene (predicted amino acid identity, 92%). The second 459-bp monoxygenase fragment (GenBank accession no. EU734589), amplified from the TMOA-F/R primer set, was closely related to the *Burkholderia cepacia* AA1 *tbhA* gene (predicted amino acid identity, 87%). Additionally, strain BD-a59 retrieved one 508-bp dioxygenase fragment with the TODC1-F/R primer set (GenBank accession no. EU734590)

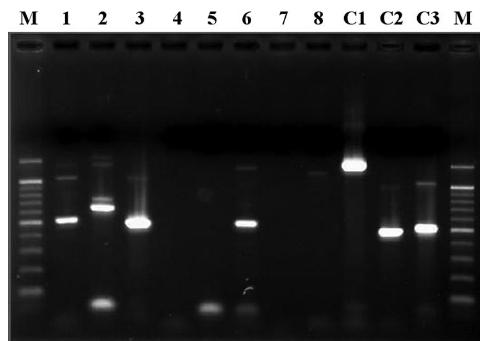

FIG. 4. PCR examination of strain BD-a59 for the presence of selected BTEX catabolic genes, using the primer sets NahAc (lane 1), TBMD (lane 2), TMOA (lane 3), TOL (lane 4), XYLA (lane 5), TODC1 (lane 6), RIESKE (lane 7), and XYLE (lane 8). Positive controls for PCR were the 16S rRNA gene of strain BD-a59 (lane C1), TOL of *P. putida* mt-2 (lane C2), and TODC1 of *R. pickettii* PKO1 (lane C3). M, molecular size marker (100-bp ladder).

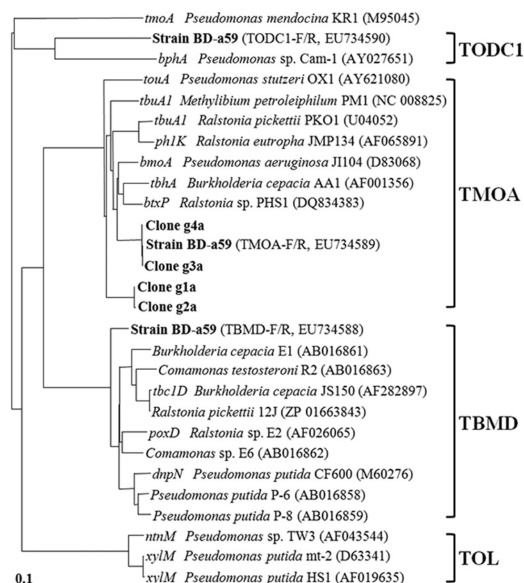

FIG. 5. A neighbor-joining tree showing the phylogenetic relationships among oxygenase gene sequences derived from strain BD-a59 and the contaminated site and corresponding oxygenase sequences of known BTEX degraders. The sequences of clones g1a, g2a, g3a, and g4s were retrieved from bacterial mRNA of the contaminated site by RT-PCR using the TMOA-F/R primer. The scale bar equals 0.1 change per nucleotide position. Representative sequences in the dendrogram obtained from GenBank were used in the phylogenetic analysis (GenBank accession numbers are shown in parentheses).

that was very similar to known dioxygenases (e.g., amino acid identity to the *Pseudomonas* sp. strain Cam-1 *bphA* gene, 96%). The TODC1-F/R primer set produced PCR amplicons from *R. pickettii* PKO1 and strain BD-a59, both of which can degrade all six BTEX compounds aerobically. However, it is not clear whether the TODC1 gene is responsible for metabolism of all six BTEX compounds in strain BD-a59, because this bacterium has three different oxygenase genes. More research on these oxygenase genes is needed to properly examine their function and regulation. Strain BD-a59 was taxonomically distant from previously characterized BTEX degraders, yet the oxygenase gene sequences retrieved from strain BD-a59 were very similar to those in other BTEX-degrading bacteria, which suggested possible lateral gene transfers (31).

RT-PCR using the TMOA-F/R primer and mRNA from the contaminated soil was applied to amplify oxygenase genes related to BTEX biodegradation by strain BD-a59 and produced the RT-PCR product successfully, which was ligated into the plasmid vector and sequenced. PCR without reverse transcriptase treatment did not produce any PCR product. The retrieved sequences (clones g1a, g2a, g3a, and g4s) were aligned, and their relationships to oxygenase genes of strain BD-a59 and other known BTEX degraders are presented in Fig. 5. The oxygenase sequences of mRNA retrieved from the contaminated site clustered into two major branches within the TMOA cluster, one of which was very closely related to the oxygenase sequence amplified by the TMOA-F/R primer set from strain BD-a59, with just one nucleotide difference. Because one nucleotide difference can be caused by PCR or sequencing biases, we concluded that the mRNA transcripts of related oxygenase genes might be derived from strain BD-a59.

In conclusion, by using a direct-plating procedure and including YE in our isolation medium, we discovered a novel bacterium belonging to the genus *Pseudoxanthomonas* that had the ability to degrade all six BTEX components. A small amount of YE significantly enhanced BTEX degradation by strain BD-a59. This nutritional need of the bacterium was also supplied by unknown components associated with sterile soil solids. Strain BD-a59 mediated BTEX degradation rapidly in a soil slurry system; thus, this bacterium may have been a prominent BTEX degrader in its native gasoline-contaminated site and shows potential in field applications for bioremediation of contaminated sites.

ACKNOWLEDGMENTS

These efforts were supported by grants from the 21C Frontier Microbial Genomics and Application Center program and the Environmental Biotechnology National Core Research Center, Ministry of Education, Science and Technology, Republic of Korea.

REFERENCES

1. **Assinder, S. J., and P. A. Williams.** 1990. The TOL plasmids: determinants of the catabolism of toluene and the xylenes. Adv. Microb. Physiol. **31:**1–69.
2. **ASTM International.** 2007. D2974-07a. Standard test methods for moisture, ash, and organic matter of peat and other organic soils. ASTM International, West Conshohocken, PA.
3. **ASTM International.** 2007. D4972-01. Standard test method for pH of soils. ASTM International, West Conshohocken, PA.
4. **ASTM International.** 2005. D4221-99. Standard test method for dispersive characteristics of clay soil by double hydrometer. ASTM International, West Conshohocken, PA.
5. **Atlas, R. M., and C. E. Cerniglia.** 1995. Bioremediation of petroleum pollutants: diversity and environmental aspects of hydrocarbon biodegradation. BioScience **45:**332–338.
6. **Baldwin, B. R., C. H. Nakatsu, and L. Nies.** 2003. Detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR. Appl. Environ. Microbiol. **69:**3350–3358.
7. **Bowlen, G. F., and D. S. Kosson.** 1995. In situ processes for bioremediation of BTEX and petroleum fuel products, p. 515–542. *In* L. Y. Young and C. E. Cerniglia (ed.), Microbial transformations and degradation of toxic organic chemicals. Wiley-Liss Inc., New York, NY.
8. **Chakraborty, R., S. M. O'Connor, E. Chan, and J. D. Coates.** 2005. Anaerobic degradation of benzene, toluene, ethylbenzene, and xylene compounds by *Dechloromonas* strain RCB. Appl. Environ. Microbiol. **71:**8649–8655.
9. **Daane, L. L., I. Harjono, G. J. Zylstra, and M. M. Häggblom.** 2001. Isolation and characterization of polycyclic aromatic hydrocarbon-degrading bacteria associated with the rhizosphere of salt marsh plants. Appl. Environ. Microbiol. **67:**2683–2691.
10. **Dean, B. J.** 1985. Recent findings on the genetic toxicology of benzene, toluene, xylenes and phenols. Mutat. Res. **145:**153–181.
11. **Eriksson, M., J.-O. Ka, and W. W. Mohn.** 2001. Effects of low temperature and freeze-thaw cycles on hydrocarbon biodegradation in arctic tundra soil. Appl. Environ. Microbiol. **67:**5107–5112.
12. **Ezaki, T., Y. Hashimoto, Y. Ashimoto, and E. Yabuuchi.** 1989. Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int. J. Syst. Bacteriol. **39:**224–229.
13. **Hatzinger, P. B., K. McCalay, S. Vainberg, M. Tugusheva, C. W. Condee, and R. J. Steffan.** 2001. Biodegradation of methyl *tert*-butyl ether by a pure bacterial culture. Appl. Environ. Microbiol. **67:**5601–5607.
14. **Hendrickx, B., H. Junca, J. Vosahlova, A. Lindner, I. Rüegg, M. Bucheli-Witschel, F. Faber, T. Egli, M. Mau, M. Schlömann, M. Brennerova, V. Brenner, D. H. Pieper, E. M. Top, W. Dejonghe, L. Bastiaens, and D. Springael.** 2006. Alternative primer sets for PCR detection of genotypes involved in bacterial aerobic BTEX degradation: distribution of the genes in BTEX degrading isolates and in subsurface soils of a BTEX contaminated industrial site. J. Microbiol. Methods **64:**250–265.
15. **Hubert, C., Y. Shen, and G. Voordouw.** 1999. Composition of toluene-degrading microbial communities from soil at different concentrations of toluene. Appl. Environ. Microbiol. **65:**3064–3070.
16. **Jahn, M. K., S. B. Haderlein, and R. U. Meckenstock.** 2005. Anaerobic degradation of benzene, toluene, ethylbenzene, and *o*-xylene in sediment-

free iron-reducing enrichment cultures. Appl. Environ. Microbiol. **71:**3355–3358.

17. **Jeon, C. O., W. Park, P. Padmanabhan, C. DeRito, J. R. Snape, and E. L. Madsen.** 2003. Discovery of a previously undescribed bacterium with distinctive dioxygenase that is responsible for in situ biodegradation in contaminated sediment. Proc. Natl. Acad. Sci. USA **100:**13591–13596.
18. **Jeon, C. O., M. Park, H.-S. Ro, W. Park, and E. L. Madsen.** 2006. The naphthalene catabolic (*nag*) genes of *Polaromonas naphthalenivorans* CJ2: evolutionary implications for two gene clusters and novel regulatory control. Appl. Environ. Microbiol. **72:**1086–1095.
19. **Kim, D., Y. S. Kim, S. K. Kim, S. W. Kim, G. J. Zylstra, Y. M. Kim, and E. Kim.** 2002. Monocyclic aromatic hydrocarbon degradation by *Rhodococcus* sp. strain DK17. Appl. Environ. Microbiol. **68:**3270–3278.
20. **Kim, J. M., and C. O. Jeon.** Isolation and characterization of a new benzene, toluene, and ethyl benzene degrading bacterium, Acinetobacter sp. B113. Curr. Microbiol, in press.
21. **Kukor, J. J., and R. H. Olsen.** 1996. Catechol 2,3-dioxygenases functional in oxygen-limited (hypoxic) environments. Appl. Environ. Microbiol. **62:**1728–1740.
22. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. Wiley, New York, NY.
23. **Lee, D. S., S. H. Ryu, H. W. Hwang, Y.-J. Kim, B. S. Chung, M. Park, J. R. Lee, S.-S Lee, and C. O. Jeon.** 2008. *Pseudoxanthomonas sacheonensis* sp. nov., isolated from BTEX-contaminated soil in Korea, and transfer of *Stenotrophomonas dokdonensis* Yoon et al. 2006 to the genus *Pseudoxanthomonas* as *Pseudoxanthomonas dokdonensis* comb. nov. and emended description of the genus *Pseudoxanthomonas*. Int. J. Syst. Evol. Microbiol. **58:**2235–2240.
24. **Lu, S., M. Park, H.-S. Ro, D. S. Lee, W. Park, and C. O. Jeon.** 2006. Analysis of microbial communities using culture-dependent and -independent approaches in an anaerobic/aerobic SBR reactor. J. Microbiol. **44:**155–161.
25. **Marc, V., J. Sabaté, C. Guasp, J. Lalucat, and A. M. Solanas.** 2005. Culture-dependent and -independent approaches establish the complexity of a PAH-degrading microbial consortium. Can. J. Microbiol. **51:**897–909.
26. **Olsen, R. H., J. J. Kukor, and B. Kaphammer.** 1994. A novel toluene-3-monooxygenase pathway cloned from *Pseudomonas pickettii* PKO1. J. Bacteriol. **176:** 3749–3756.
27. **Rosselló-Mora, R., and R. Amann.** 2001. The species concept for prokaryotes. FEMS Microbiol. Rev. **25:**39–67.
28. **Saeed, T., and M. Al Mutairi.** 1999. Chemical composition of the water-soluble fraction of the leaded gasolines in seawater. Environ. Int. **25:**117–129.
29. **Shinoda, Y., Y. Sakai, H. Uenishi, Y. Uchihashi, A. Hiraishi, H. Yukawa, H. Yurimoto, and N. Kato.** 2004. Aerobic and anaerobic toluene degradation by a newly isolated denitrifying bacterium, *Thauera* sp. strain DNT-1. Appl. Environ. Microbiol. **70:**1385–1392.
30. **Stanier, R. Y., N. J. Palleroni, and M. Doudorhoff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43:**159–271.
31. **van der Meer, J. R.** 2006. Evolution of catabolic pathways in *Pseudomonas* through gene transfer, p. 189–235. *In* J.-L. Ramos and R. C. Levesque (ed.), *Pseudomonas*, vol. 4. Springer, Dordrecht, The Netherlands.
32. **Venkateswarlu, K., and N. Sethunathan.** 1985. Enhanced degradation of carbonfuran by *Pseudomonas cepacia* and *Nocardia* sp. in the presence of growth factors. Plant Soil **84:**445–449.
33. **Wilson, M. S., C. Bakermans, and E. L. Madsen.** 1999. In situ, real-time catabolic gene expression: extraction and characterization of naphthalene dioxygenase mRNA transcripts from groundwater. Appl. Environ. Microbiol. **65:**80–87.
34. **Wolf, D. C., and H. D. Skipper.** 1994. Soil sterilization, p. 41–52. *In* R. W. Weaver, S. Angle, P. Bottomley, D. Bezdicek, S. Smith, A. Tabatabei, and A. Wollum (ed.). Methods of soil analysis, part 2. Soil Science Society of America, Madison, WI.
35. **Young, C. C., M.-J. Ho, A. B. Arun, W.-M. Chen, W.-A. Lai, F.-T. Shen, P. D. Rekha, and A. F. Yassin.** 2007. *Pseudoxanthomonas spadix* sp. nov., isolated from oil-contaminated soil. Int. J. Syst. Evol. Microbiol. **57:**1823–1827.
36. **Zylstra, G. J., W. R. McCombie, D. T. Gibson, and B. A. Finette.** 1988. Toluene degradation by *Pseudomonas putida* F1: genetic organization of the *tod* operon. Appl. Environ. Microbiol. **54:**1498–1503.
37. **Zylstra, G. J., and D. T. Gibson.** 1989. Toluene degradation by *Pseudomonas putida* F1: nucleotide sequence of the todC1C2BADE genes and their expression in *Escherichia coli*. J. Biol. Chem. **264:**14940–14946.
38. **Zylstra, G. J., and E. Kim.** 1997. Aromatic hydrocarbon degradation by *Sphingomonas yanoikuyae* B1. J. Ind. Microbiol. Biotechnol. **19:**408–414.