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An Uncultivated Nitrate-Reducing Member of the Genus *Herminiimonas* Degrades Toluene

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Stable isotope probing (SIP) is a cultivation-free methodology that provides information about the identity of microorganisms participating in assimilatory processes in complex communities. In this study, a *Herminiimonas*-related bacterium was identified as the dominant member of a denitrifying microcosm fed [^{13}C]toluene. The genome of the uncultivated toluene-degrading bacterium was obtained by applying pyrosequencing to the heavy DNA fraction. The draft genome comprised ~3.8 Mb, in 131 assembled contigs. Metabolic reconstruction of aromatic hydrocarbon (toluene, benzoate, *p*-cresol, 4-hydroxybenzoate, phenylacetate, and cyclohexane carboxylate) degradation indicated that the bacterium might specialize in anaerobic hydrocarbon degradation. This characteristic is novel for the order *Burkholderiales* within the class *Betaproteobacteria*. Under aerobic conditions, the benzoate oxidation gene cluster (BOX) system is likely involved in the degradation of benzoate via benzoyl coenzyme A. Many putative genes for aromatic hydrocarbon degradation were closely related to those in the *Rhodocyclaceae* (particularly *Aromatoleum aromaticum* EbN1) with respect to organization and sequence similarity. Putative mobile genetic elements associated with these catabolic genes were highly abundant, suggesting gene acquisition by *Herminiimonas* via horizontal gene transfer.

Toluene is a useful model compound for investigating the anaerobic metabolism of aromatic compounds (1, 2). Microorganisms that attack toluene use nitrate, Mn(IV), Fe(III), sulfate, and CO_2 as terminal electron acceptors (2, 3). Of these different acceptors, the greatest energy yield is obtained with nitrate. The majority of toluene degraders discovered to date that use nitrate as the final electron acceptor belong to the order *Rhodocyclales* within the *Betaproteobacteria*: *Azoarcus* spp. (4–6), *Aromatoleum aromaticum* EbN1 (formerly *Azoarcus* sp. EbN1) (7), *Georgfuchsia toluolica* (8), *Thauera aromatica* (9), and *Dechloromonas aromatica* RCB (10). In addition, *Magnetospirillum* sp. TS-6 within *Alphaproteobacteria* also degrades toluene using nitrate as the electron acceptor (11). Recently, four toluene-degrading bacterial strains belonging to the *Alphaproteobacteria* and *Gammaproteobacteria* were isolated from marine sediment (12), and a nonculture-based, DNA-based stable isotope probing (SIP) study reported the 16S rRNA gene sequences of [^{13}C]toluene degraders in nitrate-reducing microcosms (13). In that study, sequences related to *Comamonadaceae* bacteria and *Thauera* bacteria were retrieved from the ^{13}C-labeled heavy DNA fraction (13). The genome sequences of two of the isolated denitrifying toluene degraders, *A. aromaticum* EbN1 and *D. aromatica* RCB, are available (14–16), allowing metabolic reconstruction of anaerobic aromatic degradation pathways.

Anaerobically, toluene is activated by fumarate addition, which is mediated by benzylsuccinate synthetase. Benzylsuccinate is converted to benzoyl coenzyme A (CoA), a central intermediate in the anaerobic degradation of many aromatic hydrocarbons (2, 17). The benzene ring of benzoyl-CoA is reduced by benzoyl-CoA reductase (BCR), which is composed of four subunits (a heterotetramer of α, β, γ, and δ subunits). Based on differences in the organization of the gene clusters and gene sequence dissimilarities, BCR clusters into two groups: *bcr* and *bzd* types (18). The *bcr* type is associated with *Thauera*, *Magnetospirillum*, and *Rhodopseudomonas* (18), while the *bzd* type is associated with *Azoarcus* (19, 20).

During aerobic metabolism of aromatic compounds, benzoate is degraded by oxygenase systems; however, a novel benzoate degradation pathway that produces benzoyl-CoA as a central intermediate was discovered by Gescher et al. (21). Products of the benzoate oxidation gene cluster (BOX) convert benzoate to benzoyl-CoA. The BOX system is likely widespread: ~5% of all bacterial species for which genome sequences are available contain putative *box* genes (17). Both *bcr* and *box* cluster genes were found in the genomes of *Azoarcus evansii* (22, 23), *A. aromaticum* EbN1 (15), and *Magnetospirillum magneticum* AMB-1 (NC_007626).

Metagenomic sequencing has successfully revealed novel insights into the physiology and ecology of a variety of intriguing uncultivated microorganisms (e.g., BD1-5, OP11, OD1 bacteria, TM7, and marine group II *Euryarchaeota*) (24–26). Successful assembly of full and/or partial genomes of uncultivated microorganisms is the result of progress in both high-throughput sequencing and assembly and binning methodologies. As an example, the almost complete genome of *Methylotenera mobilis*, a novel methylotrophic bacterium, was reconstructed using a combination of SIP and metagenomic analyses (27). Those authors were able to evaluate genome completeness by examining the presence of various metabolic and housekeeping genes (27).

Received 2 December 2013 **Accepted** 11 March 2014
Published ahead of print 14 March 2014
Editor: H. L. Drake
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.03975-13.

doi:10.1128/AEM.03975-13

As exemplified by our previous discovery of a novel toluene-degrading, iron-reducing bacterium (28), metagenomic approaches have great potential for the discovery of novel microorganisms with bioremediation potential. Previous studies describing aromatic degradation under nitrate-reducing conditions using metagenomic approaches are scarce, despite many attempts to obtain and characterize axenic cultures. Here, we used metagenomic approaches that preclude some of the biases observed in cultivation-based studies in an attempt to characterize anaerobic aromatic degradation under nitrate-reducing conditions.

MATERIALS AND METHODS

Toluene-degrading microcosm experiments. Coal tar waste-contaminated sediment was gathered near South Glens Falls, NY, in November 2011, as described by Jeon et al. (29). Replicates of homogenized sediment (~8.5 g [wet weight]) were anoxically incubated in sterile 125-ml serum bottles containing 50 ml defined bicarbonate-buffered artificial freshwater medium under a headspace of N_2 (100%). The components of the artificial freshwater medium were as follows: 0.5 g liter^{-1} KCl, 0.2 g liter^{-1} KH_2PO_4, 1.0 g liter^{-1} NaCl, 0.5 g liter^{-1} NH_4Cl, 0.1 g liter^{-1} $CaCl_2{\cdot}2H_2O$, 0.4 g liter^{-1} $MgCl_2{\cdot}6H_2O$, 1 ml liter^{-1} nonchelated trace element mixture (30), 1 ml liter^{-1} vitamin solution (30), 1 ml liter^{-1} selenite-tungstate solution (31), and 1 mg liter^{-1} resazurin. For the electron acceptor, 7.5 mM $NaNO_3$ was added. The microcosm-fostering nitrate reduction conditions were designated the CN microcosm. After purging with N_2 gas, bicarbonate buffer was added to 20 mM (final concentration), and 1 mM Na_2S (final concentration) was used as a reducing agent. The final pH of the medium was adjusted to pH 7.0. To reduce toluene toxic effects during incubation, 0.5 ml of 2,2,4,4,6,8,8-heptamethylnonane (HMN) was added to each bottle as a carrier. Serum bottles were closed with butyl rubber stoppers (Bellco Glass Inc., Vineland, NJ), and 5 μl of either nonlabeled (^{12}C) toluene (Sigma-Aldrich, MO) or ring-$^{13}C_6$-labeled toluene (Cambridge Isotope Laboratories, Inc., Andover, MA) was injected through the stoppers by using a gas-tight syringe. This resulted in a reservoir of ~0.96 mM toluene in each bottle. The microcosms were incubated statically at 20°C in the dark. Five replicate bottles were prepared for each series to monitor biodegradation activity and successive time-dependent termination. Inoculum-unamended cultures, inoculum-autoclaved cultures, and cultures inoculated without nitrate (nitrate not amended) were used as controls.

Analytical methods. The time course of toluene concentration changes was measured in headspace gas samples (100 μl) by using a gas chromatograph (GC/FID 2010; Shimadzu, Japan) equipped with an Rtx-1 GC column (film thickness, 0.25 μm; inside diameter, 0.25 mm; length, 30 m; Restek, Bellefonte, PA) and a flame ionization detector. Nitrogen gas was used as carrier, with a column flow rate of 0.9 ml/min. The chromatographic conditions were as follows: injector temperature, 200°C (split ratio of 1:10); oven temperature program consisting of 40°C for 1 min, followed by an increase at a rate of 16°C/min to 145°C and then at a rate of 45°C/min to 300°C, then held for 1 min; detector temperature, 300°C (28). A nitrate test kit was used to measure nitrate concentrations (NECi, Lake Linden, MI). Measurement of the nitrite concentration was performed as described by Shinn (32). Production of $^{13}CO_2$ gas in the headspace was measured in serum bottles by using a GC fitted with an electron capture detector (GC/ECD-MS 6890A; Agilent, Santa Clara, CA). The GC was equipped with a Varian CP-PoraBOND Q capillary column (5-μm film thickness, 0.32 mm in diameter, 50-m length). The oven was isothermal, maintained at 33°C, and CO_2 concentrations were determined three times for each sample. Detailed methods for $^{13}CO_2$ determinations have been described by Kim et al. (28).

Metagenomic DNA extraction and isopycnic centrifugation. At a selected time point (8 days; 80% toluene degraded), single [^{13}C]toluene- and [^{12}C]toluene-amended bottles were sacrificed for DNA extraction. The sediment and cells were centrifuged at 8,000 rpm (6,790 × *g*) at 4°C for 20 min in a Mega17 R centrifuge (Hanil, Republic of Korea). The supernatant was filtered through a 0.2-μm-pore-sized filter (Adventec, Japan) to recover any remaining cells. Harvested cells were combined, immediately frozen, and stored at −80°C until extraction. Total genomic DNA was extracted from frozen cells by using the protocol described by Zhou et al. (33). For isopycnic centrifugation, 5 μg of extracted DNA was loaded into a CsCl solution (1 g ml^{-1}, combining an equal volume of water with an equal weight of CsCl [Sigma]) (34), and 120 μl of a 10-mg ml^{-1} ethidium bromide (EtBr) solution was added for the detection of heavy and light DNA fractions. The CsCl solution with extracted DNA was transferred to an 8.9-ml polyallomer centrifuge tube (Opti Seal; Beckman Coulter, Krefeld, Germany). The tubes were centrifuged in a type 90 Ti fixed-angle rotor using a Centrikon T-2190 ultracentrifuge (both from Beckman Coulter, Krefeld, Germany) at 55,000 rpm (187,526 × *g*) at 20°C for 36 h under vacuum (28). Two distinct bands of DNA were observed (one light [^{12}C, density of 1.55 g ml^{-1}] and one heavy [^{13}C, density of 1.59 g ml^{-1}]) in the [^{13}C]toluene-amended sample, while only one band was observed in the samples from the [^{12}C]toluene-amended bottle. The DNA bands were recovered by using a needle and syringe. The DNA was purified by extracting EtBr using 1-butanol-saturated with Tris-EDTA.

T-RFLP fingerprinting, cloning, and sequencing. DNA isolated by isopycnic centrifugation was used to amplify bacterial 16S rRNA with previously determined primers: 27F (6-carboxyfluorescein [FAM] labeled) and 1492R (35). The PCR conditions were as follows: 5 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 55°C, and 90 s at 72°C, 10 min at 72°C, and a final hold at 4°C. The PCR products were purified using a PCR purification kit (Solgent, Republic of Korea) in accordance with the manufacturer's recommendations. RsaI (New England BioLabs, Beverly, MA) was used for terminal restriction fragment length polymorphism (T-RFLP) analysis, and the fragmented DNAs were examined using an ABI 3730xL DNA analyzer. The T-RFLP profiles were analyzed using GeneMapper software (version 3.7; Applied Biosystems, Foster, CA).

The amplified 16S rRNA genes from the heavy DNA fraction were cloned using a TA cloning vector kit (RBC Bioscience, Taipei, Taiwan) and sequenced using M13F and M13R primers (36). Terminal restriction fragments (T-RFs; predicted *in silico* for representative clones) were obtained and their sequences were determined. For phylogenetic analysis, the 16S rRNA gene sequences of related taxa obtained from GenBank were aligned using the SILVA algorithm (http://www.arb-silva.de/aligner) (37). The phylogenetic tree was constructed using the neighbor-joining method with the Kimura two-parameter distance model within the MEGA 5 program (38). The bootstrap values were obtained with 1,000 replications.

Next-generation sequencing of heavy DNA. Quantified heavy fraction DNA (2 μg) was sequenced using 454 pyrosequencing (Genome Sequencer GLX system; Roche). Preparation and sequencing of samples and analytical processing were performed at the National Instrumentation Center for Environmental Management (NICEM), Republic of Korea, according to the manufacturer's instructions. To reduce the rate of chimerism, minimal filtering was performed for all reads by removing all that were shorter than 100 bp or had an average Phred quality score of <20 (39).

Microbial community composition. rRNA genes were identified from the metagenomic sequences by comparing in-house data sets with the RDP database (40). All reads that matched an rRNA gene sequence with an alignment length of ≥400 bp with a cut-off E value of 0.001 against the RDP database were collected. When possible, genus-level identifications were assigned based upon shared identity with a known species in the database.

Genome assembly and annotation. Metagenome sequencing reads were assembled using the Roche De Novo assembler (Newbler assembler v. 2.6). After assembly, 1,241 contigs (ca. 4.2 Mbp) were obtained from the heavy DNA fraction. Putative coding sequences (CDSs) were predicted with the MetaGeneAnnotator (41) and Integrative Services for Genomic Analysis. Predicted CDSs were annotated using the best BLAST hit against the NCBI nonredundant database. Ribosomal RNAs and tRNAs were

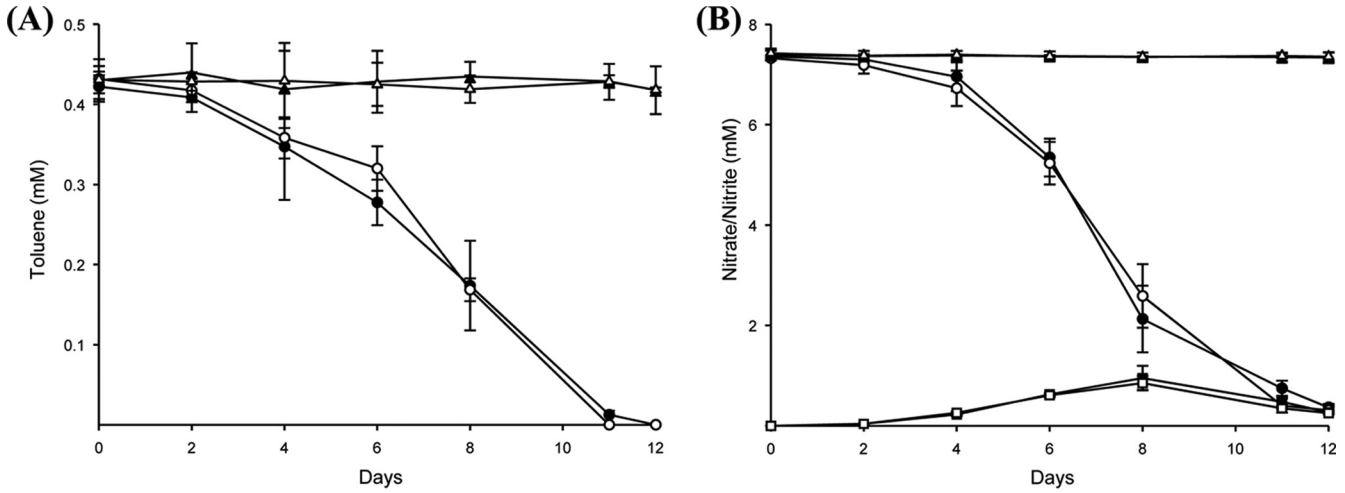

FIG 1 Toluene degradation (A) coupled to nitrate reduction (B) in the CN microcosms. Residual toluene and nitrate concentrations were measured in the [^{13}C]toluene-amended (closed circles), [^{12}C]toluene-amended (open circles), autoclaved [^{13}C]toluene-amended (closed triangles), and autoclaved [^{12}C]toluene-amended (open triangles) microcosms. Nitrite production is indicated by squares: [^{13}C]toluene-amended microcosm, closed squares; [^{12}C]toluene-amended microcosm, open squares. The error bars represent standard deviations from triplicate experiments.

identified using RNAmmer (42) and tRNAscan-SE (43), respectively. The overall GC content was determined with Artemis software (44). For binning, the oligonucleotide frequencies of the assembled contigs were calculated using the wordfreq program in the EMBOSS package (45). The GC content and frequency of betaproteobacterial genes were used to select betaproteobacterial contigs from the total 1,241 contigs. In addition, we retained only those contigs that were larger than 5 kb in order to increase taxonomic uniformity. This pipeline yielded 131 contigs that could be confidently ascribed to *Betaproteobacteria*. We also found that the binned contigs had a consistent phylogenetic profile and were hence more likely to originate from a single organism (46). Average nucleotide identity (ANI) was calculated as described previously (47). For functional assignment of the predicted genes, BLAST analysis was performed with the Clusters of Orthologous Groups, GenBank, Pfam, and Kyoto Encyclopedia of Genes and Genomes databases.

Nucleotide sequence accession numbers. The results of this metagenome shotgun project have been deposited with DDBJ/EMBL/GenBank under the accession number AVCC00000000. The version described in this paper is version AVCC01000000.

RESULTS AND DISCUSSION

Toluene degradation coupled with nitrate reduction. Toluene degradation was coupled to nitrate reduction in the microcosms (Fig. 1). Following a short lag time the toluene disappeared, and nitrate levels were reduced after 12 days of incubation. The nitrate concentration in the microcosm decreased from 7.4 mM to 0.4 mM during the incubation period, with transient accumulation of nitrite (up to 1 mM). Due to dissolution in HMN, the actual toluene concentrations were lower than the injected toluene concentration (~0.96 mM). The stoichiometry of toluene degradation with denitrification is as follows: $C_7H_8 + 7.2\ NO_3^- + 0.2\ H^+ \rightarrow 7\ HCO_3^- + 3.6\ N_2 + 0.6\ H_2O$. The actual ratio of toluene degradation relative to denitrification in our experiment was 1:7.3, which matches remarkably well to the expected ratio (1:7.2). When the microcosm was incubated with [^{13}C]toluene as the substrate, $^{13}CO_2$ production was observed (see Table S1 in the supplemental material). On a mass basis, approximately 85% of the degraded [^{13}C]toluene was converted to $^{13}CO_2$ after completion of toluene degradation in the microcosms. No toluene loss was detected in the absence of nitrate. Neither decreases in nitrate and toluene concentrations nor increases of $^{13}CO_2$ levels were observed in the autoclaved microcosms (Fig. 1; see also Table S1).

DNA-SIP and T-RFLP analysis. To identify the microorganisms involved in toluene degradation, total genomic DNA was extracted from [^{13}C]toluene-amended microcosms prior to the complete oxidation of toluene (when approximately 80 to 90% of the toluene had disappeared) to reduce the influence of cross-feeding. Total genomic DNA was separated into light (^{12}C) and heavy (^{13}C) fractions by using isopycnic ultracentrifugation. PCR-based 16S rRNA gene T-RFLP analysis was used to compare the microbial community profiles in the light and heavy DNA fractions treated with and without added nitrate. T-RFLP fingerprinting data indicated that a major peak at 117 bp was present in all toluene-amended samples (see Fig. S1 in the supplemental material). The 117-bp fragment was highly enriched (especially relative to other 16S rRNA signals) in the heavy DNA fraction (see Fig. S1B) and absent in DNA from the no-nitrate-treated samples. Clone libraries of 16S rRNA genes were constructed to identify the terminal restriction fragments (T-RFs). Sequence analysis showed that clones with a T-RF size of 117 bp were affiliated with the 16S rRNA gene sequences of the genus *Herminiimonas*, which belongs to the family *Oxalobacteraceae* within the order *Burkholderiales* (Fig. 2; see also Table S2 in the supplemental material). Although members of the order *Burkholderiales* are broadly capable of aerobically metabolizing aromatic compounds (48), to our knowledge, anaerobic aromatic metabolism has not previously been reported in this order. Of the 23 clones, 19 belonged to a single phylotype of *Herminiimonas*, sharing 99.7% 16S rRNA gene identity with each other (these were assumed to be identical, considering PCR-induced sequencing variation and possible multiple copies of rRNA genes [49] [see Table S2]). Only two clones were affiliated with *Betaproteobacteria* but not in the clades of *Oxalobacteraceae*. Sequences similar to the *Herminiimonas* 16S rRNA gene sequence (98.5% and 97.2% identity, respectively) were detected in an anthracene-SIP experiment (50) and in a radionuclide-contaminated aquifer sediment found near well GW-836 at

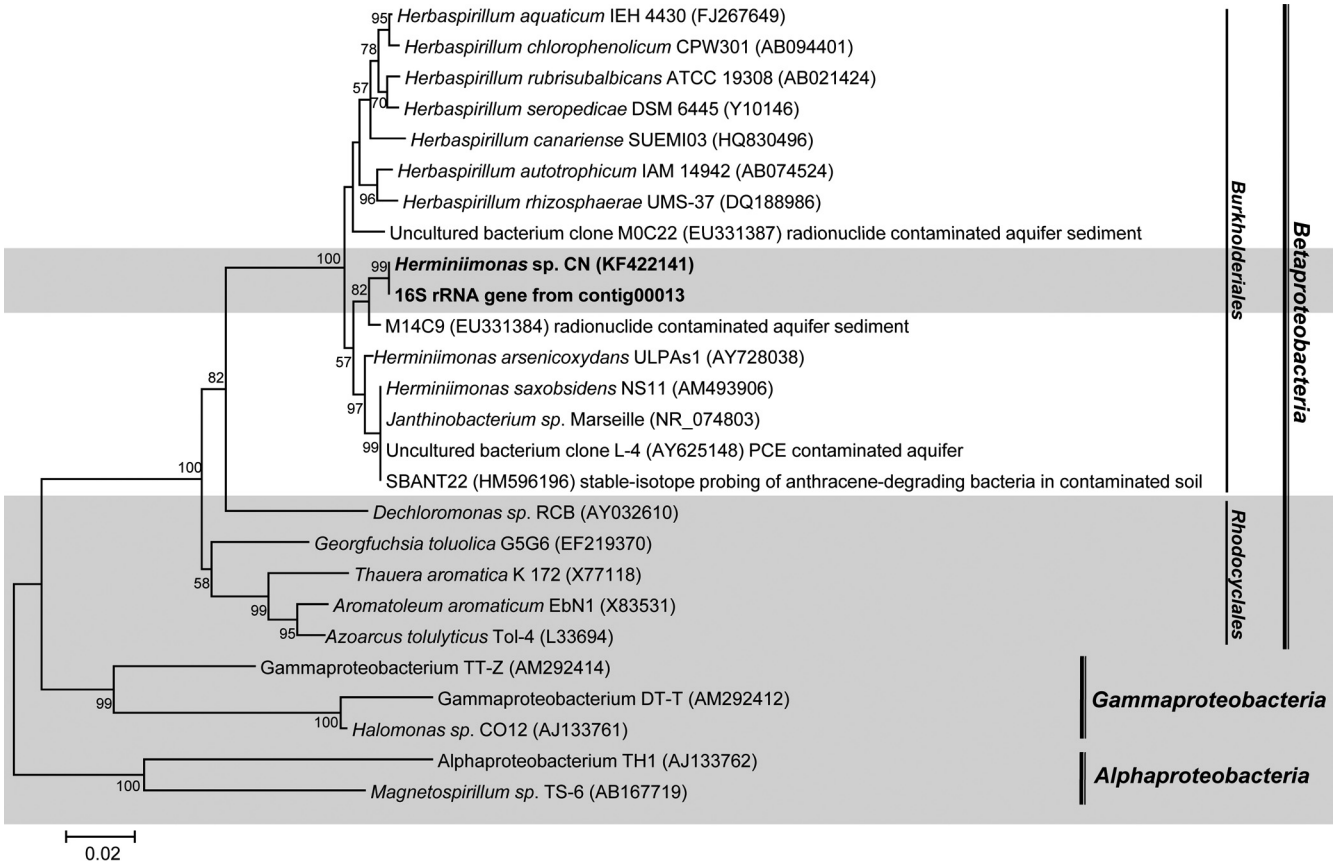

FIG 2 Phylogenetic analysis of the dominant bacterial 16S rRNA gene sequence derived from the heavy DNA fraction. The phylogenetic tree was constructed using the neighbor-joining method. The bootstrap values at each node are the percentage of 1,000 replicates. Bar, 0.2 changes per nucleotide position. Sequences highlighted with a gray box indicate denitrifying toluene degraders.

the Oak Ridge National Laboratory in Tennessee (51). The phylogenetic positions of all obtained clone sequences are shown in Fig. S2 in the supplemental material.

Metagenome sequencing of ^{13}C-labeled DNA, assembly, and binning. To characterize the genetic properties of the nitrate-reducing, toluene-degrading microorganisms, the heavy [^{13}C]DNA was sequenced using 454 pyrosequencing (Roche). A half-plate GS-FLX Titanium run yielded 683,701 reads containing 218.5 Mbp of sequence, with an average read length of 320 bp.

The microbial community composition was inferred from single raw reads of the 16S rRNA genes ($n = 293$) obtained from the metagenome data set. *Betaproteobacteria* were common (94.9%) (Table 1), with the most abundant family being *Oxalobacteraceae* (84% of the total 16S rRNA genes identified). Because the genus *Herminiimonas* belongs to the family *Oxalobacteraceae*, the tally of metagenomic 16S rRNA genes provided strong independent support of our conclusions derived from the T-RFLP analysis and clone library, namely, that *Herminiimonas* species were active in the toluene-degrading microcosm.

The assembled contigs were sorted into bins based on GC content, tetranucleotide frequency, and open reading frame (ORF) annotation. The results of the principal component analysis of tetranucleotide frequencies are shown in Fig. 3. The ORF sequences of 131 selected contigs yielded consistent BLAST hits to genes affiliated with *Betaproteobacteria* (average of 88.8% of total

TABLE 1 Taxonomic distribution of bacterial 16S rRNA gene sequences in the heavy DNA fraction obtained from the CN microcosm ($n = 293$)

Phylogenetic group	Raw read count (%)
Alphaproteobacteria	
Xanthobacteraceae	1 (0.3)
Betaproteobacteria	
Comamonadaceae	5 (1.7)
Oxalobacteraceae[a]	**246 (84.0)**
Rhodocyclaceae	1 (0.3)
Unclassified *Burkholderiales*	26 (8.9)
Deltaproteobacteria	
Bacteriovoracaceae	1 (0.3)
Gammaproteobacteria	
Chromatiaceae	1 (0.3)
Pseudomonadaceae	2 (0.7)
Flavobacteriia	
Flavobacteriaceae	3 (1.0)
Holophagae	
Holophagaceae	5 (1.7)
Unclassified *Bacteria*	2 (0.7)

[a] The dominant phylotype and its read count are shown in boldface.

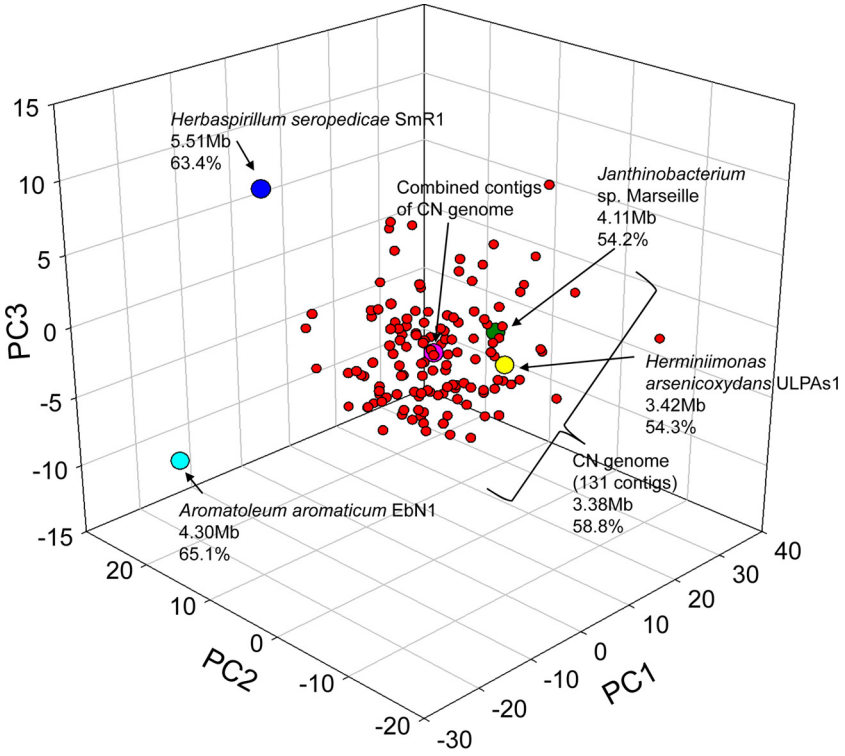

FIG 3 Principal component analysis of tetranucleotide frequencies of the CN genome and related bacterial genomes. The 131 contigs of the CN genome are shown as red circles, and the combined contigs are indicated by a pink circle. Reference genomes are indicated in yellow (*Herminiimonas arsenicoxydans* ULPAs1; NC_009138), green (*Janthinobacterium* sp. Marseille; NC_009659), blue (*Herbaspirillum seropedicae* SmR1; NC_014323), and sky blue (*Aromatoleum aromaticum* EbN1; NC_006513) circles. The genome size and G+C content are indicated below the appropriate strain name.

ORFs within each contig) and *Oxalobacteraceae* (average of 59.7% of total ORFs within each contig). The above-described analysis of T-RFLP and the clone library of the 16S rRNA genes, together with the analysis of the metagenomic raw 16S rRNA reads from [^{13}C]DNA, indicated that a single phylotype of *Betaproteobacteria* (*Oxalobacteraceae*) was dominant. Thus, the binned contigs likely originated from a single bacterium that was dominant in our microcosm.

Next, we assembled a CN draft *Herminiimonas* genome (henceforth referred to as the CN genome) for the uncultivated, toluene-degrading, nitrate-reducing bacterium. No 16S rRNA or phylogenetic genes on the contigs were affiliated with other organisms. The CN genome consisted of 131 contigs, amounting to 3.38 Mbp, with a total of 3,196 coding sequences. The mean coverage depth of the CN genome was 52.3×, with an overall GC content of 58.8%. The GC content of *Herminiimonas* strains ranges from 52.2 to 59.0%. To check the quality completeness and potential contamination of the reads contributing to the CN genome, we examined the draft genome for the presence of 107 key proteins conserved in 95% of all sequenced bacteria (26), and we detected 75 (70.1%). Furthermore, no duplicated single-copy genes were observed. We are confident that the CN genome assembly matched the dominant toluene-degrading bacterium, because the 16S rRNA gene contained in contig 00013 harbored a T-RF fragment (117 bp) matching the major peak shown in Fig. 2. Genomic comparison with *Herminiimonas arsenicoxydans* and *Herbaspirillum seropedicae* using reciprocal ANI revealed 73.7 to 74.5% identity. The broad genomic characteristics of the CN genome obtained from our metagenomic data set are compared with the genomic characteristics of three closely related bacteria in Table S3 in the supplemental material. The CN genome size was comparable with that of *H. arsenicoxydans* but was smaller than that of *H. seropedicae*. However, the total number of tRNA genes in the CN genome was lower than in both *H. arsenicoxydans* and *H. seropedicae*. These results indicate that the CN genome reported here is indeed a draft genome with an incomplete tally of genes, perhaps as a consequence of our strict binning process. Nonetheless, because a substantial proportion of the genome was successfully assembled, we proceeded to analyze aromatic hydrocarbon metabolic pathways to assess the genetic capability of this uncultivated bacterium to metabolize aromatic hydrocarbons.

Aromatic hydrocarbon degradation. Pathways for aromatic hydrocarbon compound degradation reconstructed from the CN genome are shown in the schematic in Fig. 4. The contigs harboring genes involved in the degradation of aromatic hydrocarbon compounds are summarized in Table S4 in the supplemental material.

Toluene. The clusters of putative *bss* and *bbs* genes responsible for toluene degradation were located on contig 00056 and contig 00041 of the CN genome, respectively (Fig. 5). The organization of the *bss* and *bbs* gene clusters in the CN genome was similar to that in *A. aromaticum* EbN1 and *Azoarcus* sp. CIB. However, the sequence of the key putative gene for toluene degradation, *bssA*, matched most closely to that of *Magnetospirillum* sp. TS-6 (92% amino acid similarity) within the *Alphaproteobacteria*. The putative *bssH* gene was located on another contig (contig 00037; 30.3

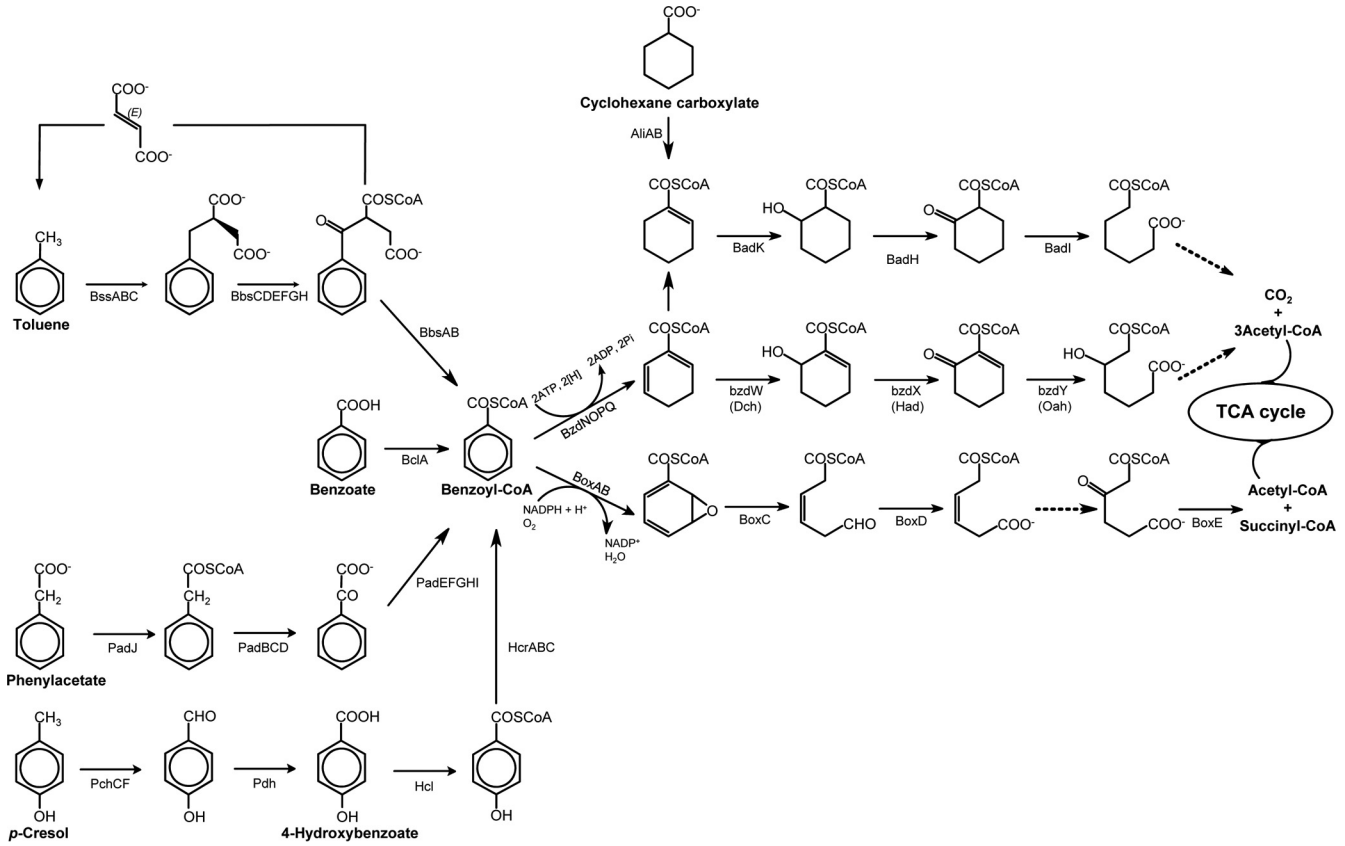

FIG 4 Aromatic hydrocarbon degradation pathways reconstructed from the CN genome. Enzymes: BclA, benzoyl-CoA ligase; BzdNOPQ, benzoyl-CoA reductase class I; Dch, dienoyl-CoA hydratase; Had, hydroxyacyl-CoA dehydrogenase; Oah, oxoacyl-CoA hydrolase; PchCF, *p*-cresol methylhydroxylase; Pdh, *p*-hydroxybenzaldehyde dehydrogenase; Hcl, 4-hydroxybenzoate-CoA ligase; HcrABC, 4-hydroxybenzoyl-CoA reductase; BssABC, benzylsuccinate synthase; BbsEF, benzylsuccinate CoA-transferase; BbsG, benzylsuccinyl-CoA dehydrogenase; BbsH, phenylitaconyl-CoA hydratase; BbsCD, alcohol dehydrogenase; BbsAB, benzoylsuccinyl-CoA thiolase; PadJ, phenylacetate-CoA ligase; PadBCD, phenylacetyl-CoA:acceptor oxidoreductase; PadEFGHI, phenylglyoxylate: NAD^+ oxidoreductase; BoxAB, benzoyl-CoA oxygenase; BoxC, benzoyl-CoA dihydrodiol lyase; BoxD, $NADP^+$-specific aldehyde dehydrogenase; BoxE, β-ketoadipyl-CoA thiolase; AliA, cyclohexanecarboxylate-CoA ligase; AliB, cyclohexanecarboxylate-CoA dehydrogenase; BadK, cyclohex-1-ene-1-carboxy-CoA hydratase; BadH, 2-hydroxycyclohexane carboxyl-CoA dehydrogenase; BadI, 2-ketocyclohexane carboxyl-CoA hydrolase. The dashed lines indicate reactions involving several steps.

kb). BssH has been suggested to export toluene or toluene-derived metabolites (e.g., benzylsuccinate) (52). Since the genomes of *Geobacter* spp. do not contain the *bssH* gene, it may be not essential for toluene degradation. In the *A. aromaticum* EbN1 genome, five characteristic genes are located downstream of *bssH*. Four of these five genes (homologs of c2B001, c2A200, c2A203, and c2A204) were conserved in contig 00056 and were located downstream of the *bss* gene cluster. However, the relevance of these genes in anaerobic toluene catabolism and whether they belong to the *bss* gene cluster remain unknown (52). As in *Geobacter metallireducens* (an iron-reducing *Deltaproteobacteria* strain), the *bbsJ* (encodes a toluene-induced protein of unknown function), and *bbsI* genes were not found in the *bbs* gene cluster of the CN genome, despite conservation of these genes in the *bss* gene clusters of *A. aromaticum* EbN1, *Azoarcus* sp. CIB, and *T. aromatica* K172.

***p*-Cresol.** The CN genome harbors single copies of the putative *pchFC* (coding for *p*-cresol methylhydroxylase) and *pdh* (coding for *p*-hydroxybenzaldehyde dehydrogenase) genes responsible for the degradation of *p*-cresol. The *pchFC* gene was identified as involved in *p*-ethylphenol methylhydroxylase activity in *A. aromaticum* EbN1 (53). However, homologs of the genes involved in the successive steps for *p*-ethylphenol degradation in *A. aromaticum* EbN1 were not found in the CN genome. Although most of the putative genes involved in aromatic compound degradation were highly similar to those of *A. aromaticum* EbN1 (see Table S4 in the supplemental material), the putative *pchFC* sequence best matched that of an autotrophic nitrite-oxidizing bacterium (71 to 80% amino acid sequence similarity), *Nitrospira defluvii* (see Table S4 and Fig. S3 in the supplemental material).

4-Hydroxybenzoate. HbcL (4-hydroxybenzoate-CoA ligase) is the initial 4-hydroxybenzoate-activating enzyme. Two putative *hbcL* copies were encoded in a single contig within the CN genome. The second step in 4-hydroxybenzoate degradation is performed by 4-hydroxybenzoyl-CoA reductase (HcrCAB), which is putatively encoded in another contig. Putative ORFs encoding maturation factor of the 4-hydroxybenzoyl-CoA reductase and 4-hydroxybenzoate transporter were located near the *hcrCAB* genes (see Fig. S4 in the supplemental material). This arrangement is similar to that for the *A. aromaticum* EbN1 genome, in which the *hcrL* and *hcrABC* genes are also not linked in a cluster. The putative *korAB* genes, which may be involved in regeneration of the ferredoxin of the BCR system, were clustered with putative

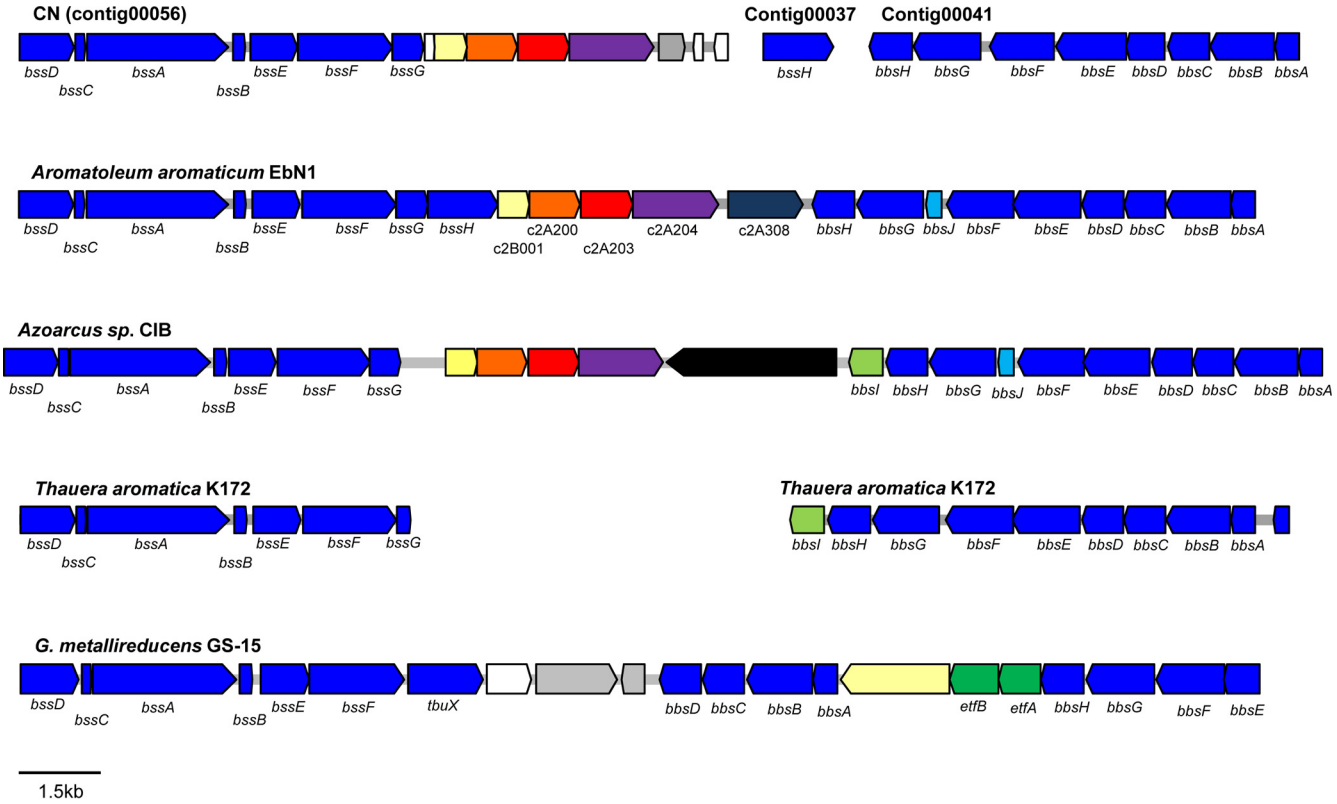

FIG 5 Comparison of toluene degradation gene clusters between the CN genome and related bacterial genomes.

hcrCAB genes (see Table S4 in the supplemental material; see also "Benzoate and benzoyl-CoA," below). According to Wöhlbrand et al., the regenerated ferredoxin may be used by both the BCR system (see below) and 4-hydroxybenzoyl-CoA reductase (54).

Phenylacetate. Phenylacetate degradation is initiated by phenylacetate-CoA ligase. Two copies of putative phenylacetate-CoA ligase genes and two sets of putative phenylacetate ABC transporter genes were identified in the CN genome (see Table S4 and Fig. S5 in the supplemental material). The putative phenylacetate ABC transporter genes were homologous to those in *Herbaspirillum* spp. and *D. aromatica* RCB (see Table S4). The CN genome included a *pad* operon, which included putative *padBCD* (encoding the phenylacetyl-CoA:acceptor oxidoreductase) and *padEFGHI* (coding for phenylglyoxylate:acceptor oxidoreductase) genes, while a putative *padB* gene was observed in another contig. An ORF homologous to thioesterase genes was observed downstream of the putative *padC* gene; the encoded protein may be involved in the enzymatic release of CoA from phenylglyoxylyl-CoA, which is formed by phenylacetyl-CoA:acceptor oxidoreductase (55). In the *A. aromaticum* EbN1 genome, the gene encoding thioesterase was located near the *padJ* gene.

Benzoate and benzoyl-CoA. The *Herminiimonas* genus has not been reported to harbor strains that degrade aromatic hydrocarbons under aerobic or anoxic conditions, and no corresponding genes have been noted in their genomes (56). However, we identified both anaerobic and aerobic benzoate degradation pathways in the CN genome (see Table S4).

As the first step of the aerobic benzoate degradation pathway in the CN genome, benzoate is likely catalyzed by benzoate-CoA ligase (encoded by a putative *bclA* gene) to benzoyl-CoA. A series of aerobic reactions catalyzed by BoxABC degrades benzoyl-CoA to acetyl-CoA and succinyl-CoA. The order of putative *box* genes in the CN genome cluster resembled that in *A. aromaticum* EbN1 (Fig. 6). However, the amino acid sequences of putative *boxBCZ* genes in the CN genome were similar to those of *Cupriavidus* spp. within the family *Burkholderiaceae* (75 to 90% similarity [see Table S4]). *A. aromaticum* EbN1 carries two putative *bclA* gene copies: one (ebA2757) is within the putative *box* gene cluster, and the other (ebA5301) is located near the *bcr* gene cluster. However, *T. aromatica* and *Magnetospirillum* strains contain only one *bclA* gene and may use the same benzoate-CoA ligase (BclA) for both the aerobic and anaerobic pathways (57, 58). While the *bclA* gene from *T. aromatica* is located in the *box* gene cluster for aerobic benzoate degradation (58), the *bclA* gene in *Magnetospirillum* strains is not linked to the *bcr* or *box* clusters (57) but is instead located within a gene cluster that encodes an ABC transporter. One putative *bclA* gene was detected in the CN genome, and its amino acid sequence was similar to ebA5301 (80%) of *A. aromaticum* EbN1. The putative *bclA* gene of the CN genome was located within a cluster of putative genes encoding a benzoate ABC transporter that matches most closely one carried by *Magnetospirillum* sp. Therefore, aryl-CoA (benzoyl-CoA) may be a common intermediate of aromatic hydrocarbon degradation under both oxygenated and anoxic conditions. This strategy is considered an adaptation by some facultative denitrifiers to fluctuating oxygenated and anoxic conditions (59). Harboring a ring reducing system that functions under both oxygenated and nitrate-reducing conditions

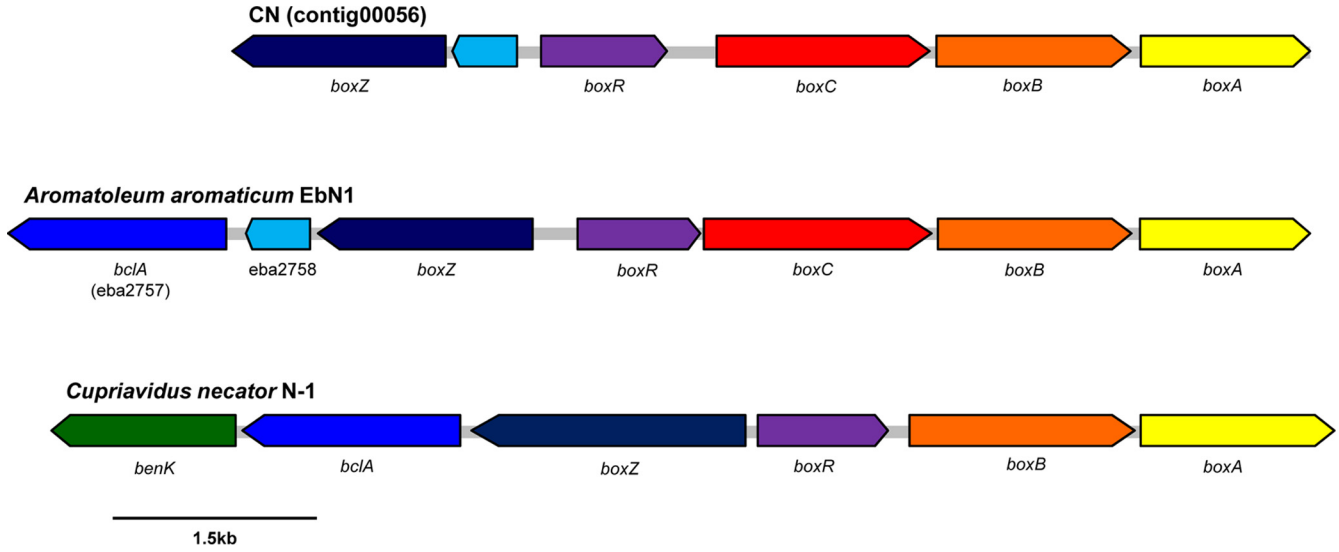

FIG 6 Gene clusters involved in the aerobic degradation of benzoyl-CoA identified in the CN, *Aromatoleum aromaticum* EbN1, and *Cupriavidus necator* N-1 genomes.

may confer an energetic advantage to strain CN, since the BOX system uses two fewer ATPs than the BCR system (see below).

Benzoyl-CoA is a central intermediate in anaerobic aromatic degradation. Benzoyl-CoA is reductively dearomatized by the BCR system under nitrate-reducing conditions. The CN genome contains all the putative genes required for the anaerobic benzoyl-CoA pathway, including those encoding four BCR subunits (*bzdNOPQ*), ferredoxin (*bzdM*), the reduced ferredoxin-regenerating system (*korAB* and *bzdV*), and a modified β-oxidation pathway (*dch*, *had*, and *oah*). Two types of ATP-dependent BCR enzymes that share an ancestor have been proposed based on amino acid sequence comparison: (i) the *bcr*-type BCR present in *T. aromatica*, *Rhodopseudomonas palustris*, and *Magnetospirillum* spp.; (ii) the *bzd*-type BCR present in *Azoarcus* spp. (18, 60). The sequence of the putative benzoyl-CoA reductase in the CN genome matched closely that of the *bzd*-type BCR. The BCR system overcomes energetic limitations by using the low-potential electron donor ferredoxin and by coupling ATP-assisted electron transfer to benzoyl-CoA. As described earlier, ORFs encoding 2-oxoglutarate:ferredoxin oxidoreductase (KGOR, regenerating reduced ferredoxins; *korAB*) are located in the CN genome near the putative *hcrCAB* instead of near BCR. Although *korABC* is encoded in a cluster in the *A. aromaticum* EbN1 genome, only putative *korAB* genes were found in the CN genome, much like the *M. magneticum* and *T. aromatica* genomes (11, 61).

After the formation of cyclohex-1-ene-1-carboxyl-CoA, degradation likely proceeds through subsequent β-oxidation-like steps. Two different β-oxidation types, the *Rhodopseudomonas* type (BadK, BadH, and BadI) and the *Thauera* type (Dch, Had, and Oah), have been reported (19). The genomes of *A. aromaticum* EbN1 and CN contain both types of gene homologs (Fig. 4; see also Table S4 in the supplemental material). The *Thauera* type and *Rhodopseudomonas* type of the β-oxidation pathways generate 3-hydroxy-pimelyl-CoA and pimelyl-CoA as the final products, respectively. These final products are further degraded into three molecules of acetyl-CoA and one molecule of CO_2, through a dicarboxylic acid β-oxidation pathway with a glutaryl-CoA dehydrogenase and a short-chain fatty acid β-oxidation pathway (2).

The genus *Herbaspirillum*, a close relative of the genus *Herminiimonas*, contains a species that harbors genes for dioxygenase systems for benzoate, benzamide, benzonitrile, hydroxyl-benzoate, and vanillate and for further aerobic degradation of the oxygenated products (62). Only two ORFs homologous to those encoding extradiol ring cleavage dioxygenase subunits were detected in the CN genome. No further aerobic degradation pathway genes for oxygenated products were detected. Thus, it appears that the canonical oxygenase-dependent aerobic degradation pathways for aromatic compounds might not be operational in the CN bacterium.

Cyclohexane carboxylate. Putative genes encoding cyclohexane carboxylate-CoA ligase (AliA) and cyclohexanecarboxyl-CoA dehydrogenase (AliB) were identified in the CN genome. These enzymes activate cyclohexane carboxylate to cyclohexenecarbonyl-CoA. Further degradation of cyclohexenecarbonyl-CoA may be facilitated by the putative *badIHR* (as in the *Rhodopseudomonas*-type β-oxidation pathway) and adjacent *aliAB* genes (see Fig. S6 in the supplemental material). Cyclohexane carboxylate degradation-related genes were also observed in *A. aromaticum* EbN1, but the amino acid sequences encoded by the corresponding CN genes were more similar to those of *Polaromonas naphthalenivorans* CJ2 and *Leptothrix cholodnii* SP 6, which fall taxonomically into the class *Burkholderiales* (see Table S4).

Horizontal gene transfer. Most of the core CN housekeeping genes showed greatest similarity to those from genomes in the same family, *Oxalobacteraceae*. However, the putative aromatic hydrocarbon degradation genes were most similar in sequence and organization to *A. aromaticum* EbN1, *Azoarcus* spp., *T. aromatica* K172, and *D. aromatica* RCB, which group taxonomically with the family *Rhodocyclaceae*. The inability of the closest relatives of the CN bacterium, *Herminiimonas*/*Herbaspirillum*, to anaerobically degrade aromatic hydrocarbons suggests that the required genes may have been acquired from unrelated taxa via horizontal gene transfer (HGT). In support of this hypothesis, we

found that putative mobile elements known to facilitate gene transfer were highly abundant in the 3.38-Mb genome of CN (25 elements/Mb). These included 18 integrases/recombinases, 21 transposases, 3 terminases, 2 resolvases, and 41 phage-related proteins. Particularly germane to this issue, putative transposase and integrase genes were directly linked with the *bss* and *bbs* gene clusters (see Table S4 in the supplemental material). *A. aromaticum* EbN1 similarly exhibits high numbers of transposon-related genes (237; 50/Mb; (14), while the genome of *Pseudomonas putida*, an aerobic aromatic degrader, contains 96 transposon-related genes in its 6.2-Mb genome (15 elements/Mb) (14). Such trends suggest that HGT is likely a widespread mechanism that confers an aromatic compound degradation ability on many host taxa. Further studies on the nature of gene transfer systems are required to fully understand the transfer and evolution of aromatic hydrocarbon metabolic pathways.

Respiratory system. Oxygen and nitrate respiratory systems were found in the CN genome (see Table S5 in the supplemental material). Putative genes for complex I (NADH:ubiquinone oxidoreductase), complex II (succinate dehydrogenase), and complex III (cytochrome bc_1 complex) in the CN genome are likely involved in oxygen and nitrate respiration. Two terminal cytochrome *c* oxidase genes were found, allowing oxygen to serve as a terminal electron acceptor. One was an aa_3-type cytochrome *c* oxidase (CoxABC) and the other was a cbb_3-type cytochrome *c* oxidase (CcoNOP). The cytochrome aa_3-type cytochrome *c* oxidase operates at high oxygen concentrations, whereas the cytochrome cbb_3-type cytochrome *c* oxidase works at low oxygen concentrations. The presence of putative terminal oxidases with different affinities for oxygen suggests that the CN genome is capable of adapting to varying external oxygen concentrations. Although the *H. arsenicoxydans* genome encodes cytochrome *bd* quinol oxidase (CydAB), which operates in low oxygen, only the putative *cydB* gene was observed in the CN genome.

To date, the *Herminiimonas* and *Herbaspirillum* genera have not been shown to harbor the complete gene set needed for denitrification. For example, the genome of *H. arsenicoxydans* does not encode nitrous oxide reductase, which is required for the final denitrification, and the only denitrification genes found in *H. seropedicae* are those encoding nitrate reductases. However, the CN genome contained putative genes encoding the four necessary enzymes that catalyze denitrification: Nar (nitrate reductase), Nir (nitrite reductase), Nor (nitric oxide reductase), and Nos (nitrous oxide reductase) (see Table S5). These genes were most similar to homologs from *Thiobacillus denitrificans* within the order *Hydrogenophilales*. Three copies of putative nitrate/nitrite transporters were present in the CN genome, two of which were located upstream of the putative *nar* gene (dissimilatory nitrate reductase) cluster, while the third was located with the assimilatory nitrite reductase (NirB) genes (see Table S5).

Detoxification systems. *H. arsenicoxydans* is resistant to arsenic (via the arsenic efflux pump encoded by *ars*) and can oxidize arsenite (56). Arsenite oxidation is also viewed as a detoxification mechanism, because arsenate is less toxic than arsenite. No putative *aox* (coding for energy-generating arsenite oxidase) gene was identified in the CN genome. Putative genes encoding an arsenic efflux pump (*arsRDACB*) were identified (see Table S6 in the supplemental material). However, a homolog of the *arsH* gene, encoding flavoprotein (unknown function), was absent. The *ars* operon of the CN genome is more similar to that of *Eshcerichia coli* (63) than to those of *H. arsenicoxydans* (see Fig. S7 in the supplemental material) or *A. aromaticum* EbN1 (15); notably, other bacteria also carry *ars* system genes derived from phylogenetically distant taxa (63). In addition to resistance to arsenic, the CN genome carries putative genes encoding proteins responsible for oxidative stress resistance (see Table S6), such as catalase and superoxide dismutase. Other putative reactive oxygen-responsive genes found in the CN genome include two thioredoxin reductases, a hydroperoxide reductase, a hemerythrin, a bacterioferritin, and a bacterioferritin comigratory protein.

Central carbon metabolism. The CN genome contained the complete set of putative genes for the Embden-Meyerhof-Parnas (EMP) and pentose phosphate pathways. However, the Entner-Doudoroff (ED) pathway may be incomplete, because no putative genes encoding 6-phosphogluconate dehydratase and 4-hydroxy-2-oxoglutarate aldolase were found; this will be resolved by genome completion. The CN genome contained all the putative genes for the tricarboxylic acid cycle and glyoxylate cycle and putative genes for the key gluconeogenesis enzymes phosphoenolpyruvate carboxykinase and pyruvate carboxylase. However, *H. arsenicoxydans* and *H. seropedicae* do not harbor a gene coding for pyruvate carboxylase. Most of the central pathway genes in the CN genome were found to be in common with members of the family *Oxalobacteraceae*.

Conclusions. The SIP-based metagenomic approach allowed us to reconstruct metabolic pathways for anaerobic degradation in a new, uncultivated, toluene-degrading denitrifying bacterium. More than 93 genes within the CN genome were devoted to the anaerobic catabolism of aromatic compounds, suggesting a possible life strategy for this organism that emphasizes assimilation of a variety of aromatic substrates. Many of the genes in strain CN, including those involved in aromatic hydrocarbon degradation and denitrification, had not previously been reported in the *Oxalobacteraceae* clade. The absence of these genes among close phylogenetic relatives, together with a high density of mobile genetic elements, suggest that HGT has likely played an important role in the evolution of the CN genome. The ability to link anaerobic degradation of aromatic hydrocarbons to denitrification is a putative key physiological feature that distinguishes the new toluene degrader from other *Herminiimonas* species.

ACKNOWLEDGMENTS

This work was supported by Mid-Career Researcher Program NRF-2013R1A2A2A05006754 and the Basic Science Research Program (2012R1A1A2A10039384) through a National Research Foundation (NRF) grant, which was funded by the Ministry of Education, Science, and Technology (MEST), National Institute of Biological Resources (NIBR) program (2013-02-055), funded by the Ministry of Environment (MOE) and Chungbuk National University Grant in 2012, and by the BK21 Plus program. E.L.M. was supported by the U.S. National Science Foundation, grant DEB-0841999.

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