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Isolation and characterization of a moderate thermophilic *Paenibacillus naphthalenovorans* strain 4B1 capable of degrading dibenzofuran from dioxin-contaminated soil in Vietnam

Le Thi Ha Thanh,[1,4] Tran Vu Ngoc Thi,[1,4] Masaki Shintani,[2,3] Ryota Moriuchi,[3] Hideo Dohra,[3] Nguyen Hoang Loc,[4,5] and Kazuhide Kimbara[1,*]

Department of Environment and Energy System, Graduate School of Science and Technology, Shizuoka University, 3-5-1 Johoku, Naka-ku, Hamamatsu, Shizuoka 432-8561, Japan,[1] Department of Bioscience, Graduate School of Science and Technology, Shizuoka University, 3-5-1 Johoku, Naka-ku, Hamamatsu, Shizuoka 432-8561, Japan,[2] Research Institute of Green Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka, Shizuoka 422-8529, Japan,[3] Department of Biology, College of Sciences, Hue University, Hue, Thua Thien Hue 530000, Viet Nam,[4] and Institute of Bioactive Compounds, College of Sciences, Hue University, Hue, Thua Thien Hue 530000, Viet Nam[5]

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A moderate thermophilic dibenzofuran (DF) degrader, strain 4B1, was isolated from dioxin-contaminated soil in Vietnam under thermophilic condition. A 16S rRNA gene sequence analysis assigned the strain to genus *Paenibacillus*. The optimum growth temperature of strain 4B1 was 45°C with a doubling time of 2.7 h in the presence of DF as a sole carbon and energy source. The rate of its growth and DF-degradation were approximately 3-fold higher than those of a reference *Paenibacillus* sp. strain. The 4B1 strain degraded 89% of 1000 mg L^{-1} DF within 48 h cultivation at the optimum temperature. TBLASTN analysis based on its draft genome sequence revealed that this strain possessed a *dbf* gene cluster. The open reading frames (*dbfA1A2RBC*) in the cluster shared 99–100% identity with those of *Paenibacillus* sp. YK5, indicating that DF was likely degraded by an angular dioxygenation pathway in strain 4B1. Four genes in the *dbf* gene cluster (*dbfA1A2BC*) were partially induced by DF, which was observed by semi-quantitative RT-PCR. Quantitative PCR analysis of *dbfA1* transcripts, encoding the alpha subunit of DF dioxygenase, indicated that *dbfA1* was expressed 4-times higher than that of strain YK5 at 45°C. These results suggest that the faster growth and degradation of DF in strain 4B1 could be due to differences in transcriptional regulation of *dbf* cluster genes.

[**Key words:** Biodegradation; *Paenibacillus*; Dibenzofuran; Moderate thermophilic; *dbf* genes]

Dioxins, which includes polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs), are a group of chemicals known to be highly toxic, carcinogenic, and persistent in the environment (1,2). The dioxin-containing herbicide Agent Orange, which was heavily used during the Vietnam war in the 1960s, continues to pose serious consequences to the environment and human beings. High levels of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [360 pg (g-dry matter)$^{-1}$], which is considered the most toxic dioxin, were measured in soil, animal tissue, and human blood in 1999, almost 4 decades after the war, in the A Luoi Valley of Vietnam (3).

Recently, bioremediation of dioxin-contaminated environments using microorganisms has become an attractive alternative to physical and chemical treatment technologies due to their low cost and the low burden they impose on the environment (4,5). Numerous studies on microorganisms capable of dioxin decomposition have been reported using dibenzofuran (DF) as a model compound, most of which are mesophilic bacteria that were either gram-negative (6–11) or high G + C gram-positive *Actinomycetes* species (12–17). Little is known about the degradation of dioxin or DF by thermophilic bacteria (18). Iida et al. (19) isolated a spore-forming bacterial strain that was known as the first DF-degrader belonging to a low G + C group (order *Bacillales*) and had its optimum growth temperature at 40–50°C. However, there was no information about its ability to convert DF under thermophilic conditions. The biodegradation of aromatic hydrocarbons with low water solubility by thermophiles is of interest as elevated temperatures during the treatment enhance the solubility and bioavailability of the pollutants leading to more efficient bioremediation. Thermophiles, predominantly *Bacilli*, have a remarkable potential for the degradation of a wide range of environmental pollutants (20–22). Vietnam's tropical and subtropical climate make it an ideal location to isolate thermophilic degraders from their soil.

This study aimed to isolate a moderate thermophilic DF degrader, characterize its properties under high temperature conditions, and identify the genes involved in DF degradation.

MATERIALS AND METHODS

Study area, soil sampling and analysis of dioxins The A So area (formerly named A Shau), located in A Luoi District, Thua Thien Hue Province, Vietnam, was a former military base occupied by US Special Forces between 1963 and 1966 where Agent Orange was stored, dispensed, and used for perimeter spraying during the

* Corresponding author. Tel./fax: +81 53 478 1170.
E-mail address: kimbara.kazuhide@shizuoka.ac.jp (K. Kimbara).

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Vietnam conflict (3). A total of 15 soil samples were collected near this former airbase (N16°7′15″, E107°19′40″) from five sites and different depths (from 10 to 40 cm in depth) by using a stainless steel soil corer. All samples were deposited in sterile polythene bags and stored at room temperature in the dark until use in the laboratory. Soil samples were forwarded to Environmental Control Center Co. Ltd., Tokyo, Japan. Analysis of dioxins and dioxin-related compounds was performed according to a manual of dioxin analysis of soil samples, which was approved by the Ministry of Environment, Japan. PCDDs and PCDFs were analyzed by high resolution gas chromatography with high resolution mass spectrometric detection (HR GD/HR MS). Total toxic equivalence (TEQ) was calculated using the toxic equivalency factor (TEF, WHO-TEF2006).

Bacteria, media and chemicals The DF degraders isolated in this study and *Paenibacillus* sp. strain YK5 (JCM 16163) were cultured on Luria–Bertani (LB) medium (23) or mineral salt medium (W medium) (24) with an appropriate carbon source. The composition of W medium was as follows: 1.7 g L^{-1} KH_2PO_4, 9.8 g L^{-1} Na_2HPO_4, 1.0 g L^{-1} $(NH_4)SO_4$ g L^{-1}, 0.1 g L^{-1} $MgSO_4{\cdot}7H_2O$, 5 mg L^{-1} $FeSO_4{\cdot}7H_2O$ and the trace element solution consisted of 10.75 mg L^{-1} MgO, 2.0 mg L^{-1} $CaCO_3$, 4.5 mg L^{-1} $FeSO_4{\cdot}7H_2O$, 1.44 mg L^{-1} $ZnSO_4{\cdot}7H_2O$, 1.12 mg L^{-1} $MnCl_2{\cdot}5H_2O$, 0.25 mg L^{-1} $CuSO_4{\cdot}5H_2O$, 0.28 mg L^{-1} $CoSO_4{\cdot}7H_2O$, 0.06 mg L^{-1} H_3BO_3 were dissolved with concentrated HCl (51.3 μL L^{-1}). Carbon sources used in this study were: 0.1% dibenzofuran (DF) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), 0.5% sodium succinate (Wako Pure Chemicals, Osaka, Japan). *Escherichia coli* JM109 (RBC Bioscience, New Taipei City, Taiwan) used for cloning experiments was cultured at 37°C on LB medium. The final concentrations of ampicillin (Ap), isopropyl β-D-1-thiogalactopyranoside (IPTG), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) were 100 μg mL^{-1}, 0.1 μM, and 20 μg mL^{-1}, respectively.

Isolation of DF-degrading bacterium Soil samples (0.5 g) were added to 3 mL of W liquid medium supplemented with DF crystal [0.1% (w v^{-1})] as a sole carbon and energy source and incubated at 50°C on a shaker at 180 rpm. After 7 days, 10% of the culture was transferred to fresh medium and incubated under the same conditions until certain microbes grew in the medium (evaluated by the turbidity and color of the medium). After several enrichments, the diluted cultures were spread onto solid nutrient media (1/3 LB) and incubated at 50°C for several days. Each obtained colony was streaked separately onto 1/3 LB plates for purification. Different colony morphotypes were chosen and streaked onto W solid medium (with 0.005% yeast extract and 1.5% agar) supplemented with DF crystals placed on the Petri dish lid, thus allowing vapors to reach the microorganisms, and the incubation continued at the same temperature. The obtained colonies were then screened for their ability to utilize DF as a sole carbon source on the same liquid medium. Four colonies, which efficiently grew on DF at 50°C, were selected for further identification.

Standard DNA manipulation Total DNA of *Paenibacillus* strains grown overnight in 3 mL of LB medium were prepared using NucleoSpin Tissue Kit (Macherey–Nagel, Düren, Germany). The 16S rRNA genes of the isolates were amplified using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) with Ex Taq DNA polymerase (Takara Bio Inc., Shiga, Japan). The PCR amplification was performed under the following conditions: an initial step at 95°C for 2 min, followed by 35 cycles of 2 min at 95°C, 30 s at 42°C, 4 min at 72°C; and a final extension for 10 min at 72°C. The amplicons were then purified using Hi Yield Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience) and cloned with T-vector pMD20 (Takara Bio Inc.) and *E. coli* JM109 for DNA sequencing.

Identification of DF-degraders The identification of the DF-degraders was performed with their 16S rRNA gene sequences by using the EzBioCloud web server (https://www.ezbiocloud.net/). The 16S rRNA gene sequences were aligned in the ClustalW implementation of MEGA v7.0 software (25), and a neighbor-joining phylogenetic tree of the DF degrader 16S rRNA gene sequences with those of representative bacteria was constructed using the bootstrap test based on 1000 replicates.

Growth rate and DF degradation activity of growing cells Pre-cultures of strain 4B1, a representative degrader, and *Paenibacillus* sp. strain YK5 were prepared by culturing cells in 250-mL Erlenmeyer flasks containing 50 mL LB medium, incubated at 50°C on a rotary shaker at 180 rpm until the mid-log phase. These pre-cultures were inoculated into 100 mL W liquid medium containing 0.1% DF in 500-mL baffled flasks. The initial turbidity at 600 nm (OD_{600}) was set to 0.03 and incubated at 30–55°C. Bacterial growth was monitored as increasing turbidity at 600 nm until the stationary phase. Controls were cultured in the W medium without DF. The generation time was calculated based on the $\log_{10}$ (OD_{600}) of the obtained greatest slope defined as the maximum growth rate ($\log_{10}(OD_{600})$ h^{-1}).

Biodegradation of DF was tested in the W medium with DF at 45°C, whose initial OD_{600} was 0.03. Samples without bacteria were used as negative controls. At each sampling point, 50 mL of triplicate samples were taken and the remaining DF in the medium was extracted with half volume of ethyl acetate followed by dehydrate with sodium sulfate. About 1 mL of each extract was quantified using gas chromatography (GC) Shimadzu GC-2014 (Shimadzu, Kyoto, Japan) equipped with a capillary column Agilent J&W GC column DB-1 [Agilent Technologies, Santa Clara, CA, USA: 30 m by 0.32 mm (inside diameter); 0.25 μm film thickness]. The GC conditions were as follows: automatic injection mode; 320°C detector temperature; initial column temperature of 80°C for 2 min, increased to 170°C at a rate of 5°C min^{-1}, then to 300°C at 50°C min^{-1} rate, and then held for 2 min. GC data were analyzed using LAsoft Chroma Data System and CDS-Lite version 5.0 (LAsoft, Chiba, Japan).

Genome sequencing and screening for DF degradative genes The total DNA of the degrader was subjected to genomic sequencing using a high-throughput MiSeq platform (Illumina, San Diego, CA, USA). A 301-bp paired-end library was constructed using the TruSeq DNA PCR-free library preparation kit (Illumina). The obtained raw reads were filtered with Trimmomatic version 0.33 (26) as previously reported (27). The cleaned 1,279,859 high-quality read pairs, corresponding to an approximately 137-fold coverage of the genome, were assembled using the SPAdes Genome Assembler version 3.10.1 (28) with options (–careful, –only-assembler, and –cov-cutoff auto) and a default set of *k*-mer sizes. The obtained contigs were manually curated. The DFAST-core stand-alone program version 1.1.2 (29) was used for predicting open reading frames (ORFs), ribosomal RNA genes, and transfer RNA genes. Annotation was performed with the genus *Paenibacillus* protein database constructed with a utility script bundled in the DFAST-core. Average nucleotide identity (ANI) values between draft genome of isolated DF-degrader and complete genome sequences of reference strains were analyzed using EzBioCloud web server (http://www.ezbiocloud.net/). Genome-to-Genome Distance Calculator (GGDC) web server version 2.1 (http://ggdc.dsmz.de) was used to calculate the digital DNA–DNA hybridization (DDH) between these strains. The assembled contigs were screened for the presence of catabolic genes involved in DF degradation using NCBI BLAST+ version 2.2.26 with appropriate query sequences. Further putative functional gene identification was performed using the Kyoto Encyclopedia for Gene and Genomes (KEGG) database (http://www.genome.jp/kegg). Geneious Prime 2018 and Easyfig version 2.2.2 (30) were used for generating sequence alignments of the isolated sequences against those of related strains.

Extraction of RNA, cDNA synthesis, and quantitative reverse transcription (RT)-PCR To investigate the induction of DF degradative genes by DF, the DF degrader was cultivated in 100 mL of W medium containing DF [0.1% (w/v)] or succinate [0.5% (w/v)] at 45°C to the late-log phase (20–22 h). Cells were harvested by centrifugation, washed with phosphate buffered saline (PBS), and treated with RNAprotect Bacteria Reagent (Qiagen, Venlo, The Netherlands) to stabilize the RNA.

To compare the expression level of degradative genes, the obtained strain and *Paenibacillus* sp. strain YK5 were pre-cultured in 100 mL of LB medium in 500-mL flasks at 45°C until the late-log phase. The cells were then harvested by centrifugation and resuspended in the same volume of W liquid medium. Afterwards, 0.1% (w/v) of DF was added to the resultant cell suspension and incubated at 45°C. For the RNA extractions, 20 mL of culture was harvested at 0, 1 and 3 h after the cell inoculation, and similarly treated with the RNAprotect Bacteria Reagent (Qiagen).

Total RNAs were extracted by NucleoSpin RNA Isolation Kit (Macherey–Nagel). The resultant RNA samples were treated with RNase-free DNase (Promega, Madison, WI, USA) and then repurified with the Nucleospin RNA Binding Column (Macherey–Nagel). Complementary DNA (cDNA) synthesis was performed using 200 U of SuperScript III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA) with 2 μg of the total RNA and 250 ng of random hexamer primers (Invitrogen). The RNA and random primers were denatured at 65°C for 10 min. The remaining reagents [1 × First Strand Buffer, 40 U of RNase Out, 5 mM dithiothreitol (DTT), and 0.5 mM deoxynucleoside triphosphates (dNTP)] were added and the mixture was incubated at 25°C for 5 min, 50°C for 60 min, and held at 70°C for 15 min for enzyme inactivation. Specific PCR primers for the *dbfA1A2BC* genes were designed by the program Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) (Table S1) and the PCR with cDNA was performed with PrimeSTAR GXL DNA polymerase (Takara Bio) as follows: 30 cycles of amplification at 98°C for 10s, followed by 60°C for 15 s, and 68°C for 1 min. A quantitative RT-PCR experiment was performed using a PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and ABI StepOne Real-time PCR Systems (Applied Biosystems, Foster City, CA, USA) with cDNA synthesized from 2 μg of total RNA. The primers used for qRT-PCR were designed using the same Primer3 program (Table S1), and the amplified products were approximately 100 bp in length. Reaction mixture (10 μL) contained 5 μL of 2 × PowerUp SYBR Green Master Mix, 500 nM of specific primers and cDNA. The PCR conditions were: 50°C for 2 min, 95°C for 10 min; followed by 40 cycles of 95°C for 15 s, 60°C for 1 min; and a final stage at 95°C for 15 s, 66°C for 1 min, 95°C for 15 s. A serial logarithmic dilution of known amounts of 10^{-4}–1 pg μL^{-1} of the PCR products of *dbfA1* gene and 16S rRNA gene was prepared to generate standard curves. The 16S rRNA gene was used as the internal standard for normalization of RNA samples.

Nucleotide sequence accession numbers The 16S rRNA gene sequence and all the contigs of draft genome sequences including *dbf* genes of the isolate were deposited into the DNA Database of Japan (DDBJ) under accession numbers LC479451 and BJCS01000001–BJCS01000057.

RESULTS AND DISCUSSION

Isolation and identification of DF-degrading bacteria Soil samples collected from the A So area had a total amount of PCDDs ranging from 690 pg (g-dry matter)$^{-1}$ in the topsoil to 490 pg (g-dry matter)$^{-1}$ in the subsoil. The TEQ values for the topsoil and subsoil samples were 21 and 22 pg (g-dry matter)$^{-1}$, respectively,

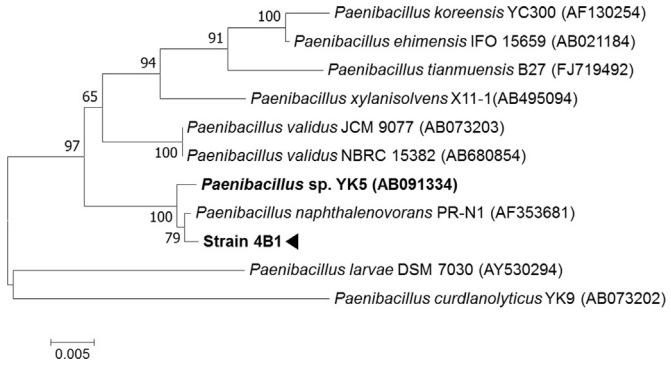

FIG. 1. Dendrogram showing the relationship of the 16S rRNA gene sequence of strain 4B1 (1297 bp of the cloned sequence, which was identical with the 16S rRNA gene sequence of the contig#41, accession no. BJCS01000041) with those reported for species of the genus *Paenibacillus*. The phylogenetic tree was constructed using the neighbor-joining method. Bootstrap percentages, based on 1000 replicates, are given at the nodes. The scale bar indicates 0.005 substitution per nucleotide position. GenBank accession numbers used for phylogenetic analysis are in parentheses. Bacteria capable of degrading DF are in boldface.

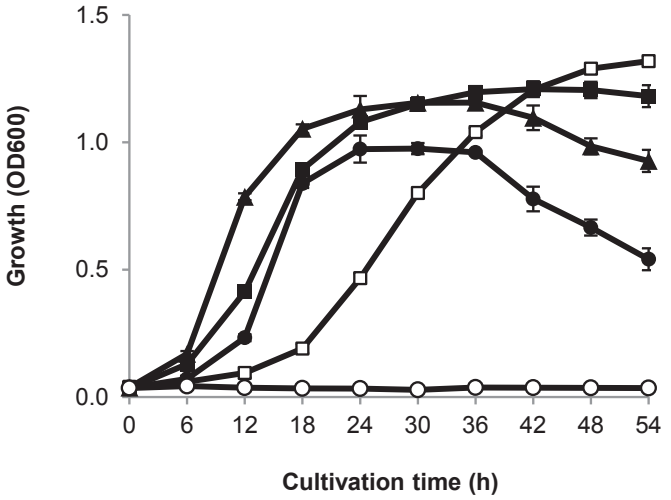

FIG. 2. Utilization of dibenzofuran (0.1%) as a sole carbon and energy source by strain 4B1 at different temperatures: 30°C (open squares), 40°C (closed squares), 45°C (closed triangles), 50°C (closed circles), 55°C (open circles). Bacterial growth was measured by OD_{600}. Each plotted value is the mean of biological triplicates with standard deviation.

which correspond with the same concentration of TCDD. These dioxin-contaminated soil samples were used for isolation of DF-degrading bacteria by the enrichment method with DF as a sole carbon and energy source at 50°C. Four strains that showed growth on DF were isolated as DF-degraders from two different soil samples. These strains formed cream-colored, smooth, circular colonies on 1/3 LB agar plates. Phylogenetic analysis of 16S rRNA gene sequences of the four degraders revealed that these strains showed closest homology with members of the genus *Paenibacillus*, which was known as a bacterial group that includes several degraders of aromatic hydrocarbons (9,31–33). We selected one dominant strain, which showed rapid growth with DF at 50°C, and designated it as *Paenibacillus* sp. strain 4B1 for further characterization. In particular, the 16S rRNA gene of strain 4B1 (approximately 1.5 kb) showed high similarity to a naphthalene degrader *P. naphthalenovorans* PR-N1 (similarity of 99.4%), and a DF degrader *Paenibacillus* sp. strain YK5 (99.1% identity) (19,34) (Fig. 1).

Features in growth with DF of *Paenibacillus* sp. strain 4B1 The temperature-dependent growth rates of strain 4B1 were investigated using 0.1% DF as the sole carbon source. Strain 4B1 was able to grow in the presence of DF from 30°C to 50°C, and there was a slight growth at 55°C in LB medium (OD_{600} was approximately 0.3). The maximum growth rate of strain 4B1 at 45°C (the doubling time in exponential growth was 2.7 h) was double that of growth at 30°C (6 h), although strain 4B1 reached a higher maximum OD_{600} at 30°C (Fig. 2). Several thermophilic *Paenibacillus* strains have the ability to degrade aromatic hydrocarbons, such as naphthalene [*Paenibacillus* sp. ORNaP1 (32), *Paenibacillus* sp. 1C (35)], dibenzothiophene [*P. naphthalenovorans* 32O-Y (33), *Paenibacillus* sp. A11-2 (36)], and phenanthrene [*Paenibacillus* sp. PHE-3 (37)]. However, only *Paenibacillus* sp. strain YK5 was known as a DF-degrading bacterium belonging to the *Paenibacillus* genus, which shows optimum growth at high temperature (40–50°C) on (nutrient rich) TSB medium (19). It should be noted that strain YK5 was able to degrade DF at 37°C but that its degradative ability was not investigated at higher temperature (19).

The growth rate and DF degradation of strain 4B1 were compared with those of *Paenibacillus* sp. strain YK5 at their optimum growth temperature (45°C). Two strains showed similar growth rate when nutrient medium (LB) was used. The doubling time of strain 4B1 was 1.38 h/generation, while strain YK5 showed the growth rate of 1.49 h/generation. Meanwhile, higher growth rate and DF degradation efficiency were observed in strain 4B1 than in strain YK5 in the presence of DF as a carbon source (Fig. 3). Strain 4B1 showed a short lag phase and a maximum growth rate of 2.4 h/generation, while strain YK5 went through a long lag phase and showed an approximately 3-fold lower doubling time (7 h) during exponential growth (Fig. 3A). The corresponding decrease in DF concentration was observed in the cultures of both strains (Fig. 3B). After 48 h, strain 4B1 degraded DF from an initial concentration of 1000 mg L^{-1} to a residual concentration of about 110 mg L^{-1} (89% of DF was degraded), whereas strain YK5 showed a slower DF degradation rate with a residue of 65% DF (650 mg L^{-1}) after 48 h of incubation (Fig. 3B).

Previous studies of bacterial growth concomitant with decreasing substrate showed that *Paenibacillus* sp. strain TSY30 completely degraded 17 mg L^{-1} DF within 100 h incubation at 30°C (9). After 120 h cultivation at 30°C, 100 mg L^{-1} of DF was almost completely degraded by *Janibacter terrae* XJ-1 (doubling time of 12 h) (14). Approximately 94% of DF (1000 mg L^{-1}) was degraded within 96 h by *Janibacter* sp. strain YY-1 at 30°C (doubling time of 13 h) (38). In comparisons with these previous data, strain 4B1 had higher growth rate and DF degradation rate in higher temperatures.

Identification of DF-degradative genes in strain 4B1 The draft genome sequence of strain 4B1 was determined using the MiSeq platform to identify its DF-degradation pathway(s). The obtained draft genome sequence consisted of 57 contigs, with a total length of 4.96 Mb and an average G + C content of 50.2 mol %. A total of 4850 coding sequences, 92 tRNAs, and 10 rRNAs were annotated in the 4B1 genome. Average nucleotide identity (ANI) values for the strain 4B1 genome against *P. naphthalenovorans* 32O-Y (CP013652), *Paenibacillus mucilaginosus* 3016 (CP003235), *Paenibacillus beijingensis* DSM 24997 (CP011058), and *Paenibacillus terrae* HPL-003 (CP003107) were 99.62%, 72.84%, 69.56%, and 68.57%, respectively (Table S2). Notably, the ANI between strain 4B1 and *P. naphthalenovorans* 32O-Y was higher than the minimum threshold for species delimitation (96.5%) (39), which suggests that they might be assigned to the same species. Digital DNA–DNA hybridization among these strains based on the Genome Blast Distance Phylogeny (GBDP) approach (Table S3), using formula 2 as

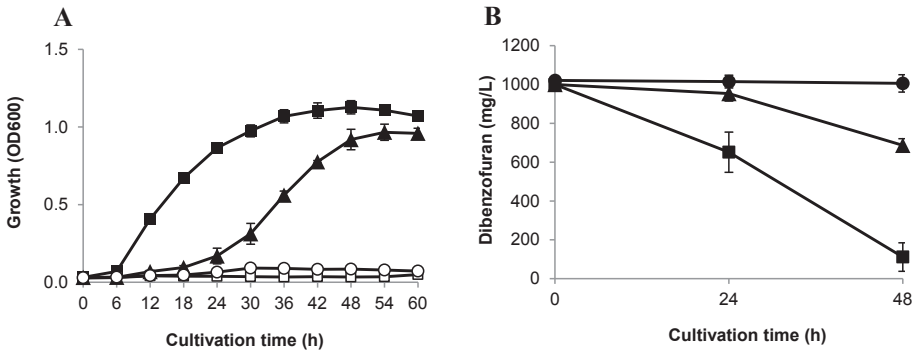

FIG. 3. (A) Growth of strain 4B1 (closed squares) and *Paenibacillus* sp. strain YK5 (closed triangles) on DF at 45°C. Controls cultures of strains 4B1 (open squares) and YK5 (open circles) were grown without DF. (B) DF degradation by growing cultures of strain 4B1 (closed squares) and strain YK5 (closed triangles). Control sample (closed circles) was incubated without bacteria. Each plotted value is the mean of biological triplicates with standard deviation.

recommended by GGDC, revealed that strain 4B1 showed high relatedness with *P. naphthalenovorans* 32O-Y (97.5%) and was a distinct species with other strains [*P. mucilaginosus* 3016 (20.8%), *P. beijingensis* DSM 24997 (23%), *P. terrae* HPL-003 (33.1%)]. The *in silico* DDH value of strain 4B1 against *P. naphthalenovorans* 32O-Y was higher than the cut-off point of 70% DDH for species delineation (40,41). These results indicate that strain 4B1 could be considered to be the same species with *P. naphthalenovorans* 32O-Y, the dibenzothiophene-utilizing bacterium (33) (hereafter referred to as *P. naphthalenovorans* strain 4B1).

TBLASTN searches for sequences involved in DF degradation in the genome of strain 4B1 were performed using amino acid sequences of DF-degrading enzymes from two representative degraders as queries: DbfA1A2BC of *Paenibacillus* sp. YK5 (AB201843), as well as DfdA1A2A3A4 and DbfBC genes of *Terrabacter* sp. DBF63 (AB471919, AB004563). The TBLASTN results are shown in Table S4. Five coding sequences (CDSs) were found as putative DF-degradative genes located on contig #2 of the 4B1 genome sharing high identity with those genes of strain YK5 (Table 1 and Fig. S1). It should be noted that the functions of the *dbfA1A2BC* gene products of YK5 were experimentally confirmed (19). The deduced amino acid sequences of locus_10160–10200 located on contig #2 showed extremely high identity (99–100%) to those of the *dbf* gene cluster of strain YK5 (19). These CDSs were named *dbfBCRA1A2*, and the *dbfA1*, *dbfA2*, and *dbfR* genes shared 100% identities with those in YK5 at the nucleotide sequence level, while *dbfB* and *dbfC* each showed a single nucleotide difference from those of strain YK5. They showed lower identities (32–60% at the amino-acid level) with the corresponding homologues involved in DF degradation of *Terrabacter* sp. DBF63, *Rhodococcus* sp. RHA1, *Rhodococcus* sp. p52, and *Rhodococcus* sp. YK2 (Table 1).

In contrast, putative genes for the ferredoxin and reductase components of the DF-dioxygenase system (DbfA3 and DbfA4) were not found near the *dbf* gene cluster of strains 4B1 (Table S4) or YK5 (19). These components are involved in the electron transfer in the dioxygenase system, which were widely found in the DF-degrading bacteria *Terrabacter* sp. DBF63, *Rhodococcus* sp. YK2, and *Nocardioides* sp. DF412 (42–44). Several degraders of aromatic compounds have the genes encoding the electron transfer system of their dioxygenase not clustered with the dioxygenase genes, but rather are located on distinct and separate genome segments. These include the dibenzo-*p*-dioxin degrader *Sphingomonas wittichi* strain RW1 (45) and the carbazole-degrader *Novosphingobium* sp. KA1 (46,47). Therefore, it is possible that the electron-transfer proteins for the dioxygenase in *Peanibacillus* are located on different contigs, although they were not currently identified.

The *dbf* gene cluster of strain 4B1 and a proposed upper-pathway of DF degradation are shown in Fig. 4. Strain 4B1 might degrade DF via an angular dioxygenation catalyzed by a dibenzofuran 4,4a-dioxygenase (DbfA), then converted to 2,2',3-trihydroxybiphenyl (THB), which is *meta*-cleaved by an extradiol dioxygenase (DbfB), and subsequently hydrolyzed to salicylate by DbfC. Notably, a yellow color developed in the culture of strain 4B1 from 12 h to the end of cultivation, while a similar color appeared later during growth of strain YK5 (30–36 h). These phenomena could support the presence of the *meta*-cleavage pathway in these strains during metabolism of DF (1).

Transcriptional analysis of *dbf* genes To understand whether the *dbf* genes were expressed in response to DF under thermophilic condition, RT-PCR analyses were performed with total

TABLE 1. Genes involved in DF degradation pathway of *P. naphthalenovorans* strain 4B1 and their products.

Locus	Gene	Position in contig	Homologue	Identity (%)[a]	Accession number[b]	Deduced function
Locus_10160	*dbfB*	430460–431422	DbfB *Paenibacillus* sp. YK5	100	AB201843	Extradiol dioxygenase
			DbfB *Terrabacter* sp. DBF63	36	AB004563	
			BphC *Geobacillus* sp. JF8	60	AB092521	
Locus_10170	*dbfC*	431473–432318	DbfC *Paenibacillus* sp. YK5	99	AB201843	Hydrolase
			DbfC *Terrabacter* sp. DBF63	35	AB004563	
			BphD *Rhodococcus* sp. RHA1	42	AB120955	
Locus_10180	*dbfR*	432308–433006	DbfR *Paenibacillus* sp. YK5	100	AB201843	Transcriptional regulator
			BphD *Geobacillus* sp. JF8	33	AB113649	
Locus_10190	*dbfA1*	433056–434351	DbfA1 *Paenibacillus* sp. YK5	100	AB201843	Alpha subunit of dibenzofuran dioxygenase
			DbfA1 *Rhodococcus* sp. p52	60	JN185320	
			DfA1 *Terrabacter* sp. DBF63	31	AB054975	
Locus_10200	*dbfA2*	434344–434847	DbfA2 *Paenibacillus* sp. YK5	100	AB201843	Beta subunit of dibenzofuran dioxygenase
			DfA2 *Terrabacter* sp. DBF63	32	AB054975	
			DbfA2 *Rhodococcus* sp. YK2	52	AB070456	

[a] Percent identity of deduced amino acid sequences.
[b] Nucleotide sequence accession numbers in GenBank databases.

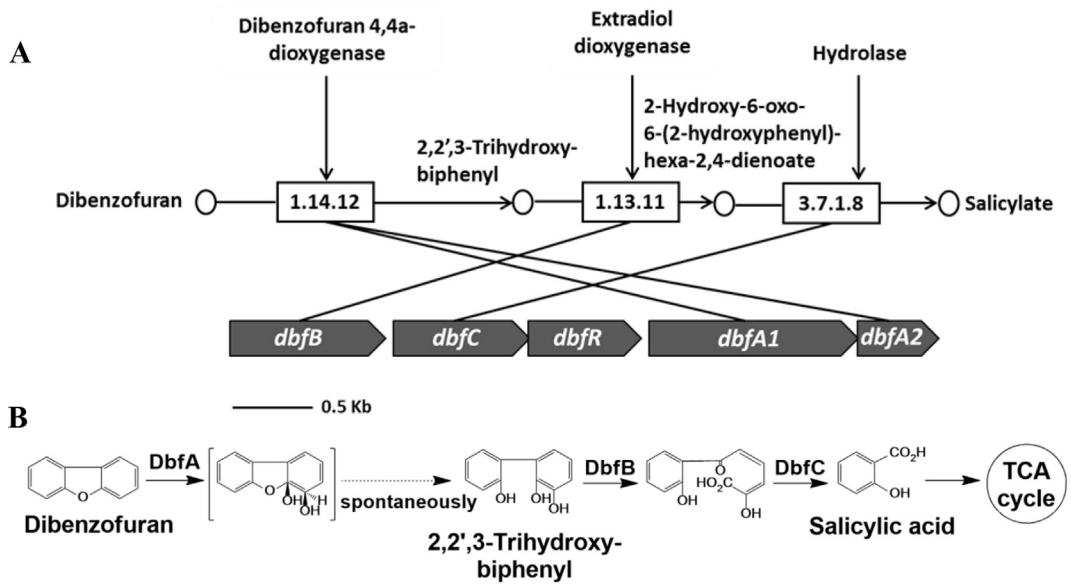

FIG. 4. (A) The *dbf* gene cluster organization of *P. naphthalenovorans* strain 4B1 involved in DF degradation with the corresponding encoded enzymes. Genes are represented by arrows, numbers in black squares are EC (Enzyme Commission) numbers from KEGG pathway database. (B) Proposed DF degradative upper pathway of strain 4B1.

RNA of strain 4B1 that grew on DF or succinate as a sole carbon source at 45°C. Fig. 5 shows amplicons of partial DNA regions of each *dbf* gene of strain 4B1 (around 500 bp). The signals of four amplicons observed from DF-cultivated cells (lane 1) were slightly stronger than those of succinate-grown cells (lane 2). These results indicate that the *dbf* genes were constitutively transcribed, and were partially induced in the presence of DF in strain 4B1. In contrast, transcription of *dbfA1* gene of strain YK5 was not observed in glucose-grown cells by Northern blot analysis, whereas those in DF-grown cells were clearly detected (19). Although strains 4B1 and YK5 have an almost identical *dbf* gene cluster, their transcriptions are probably differently regulated in each strain. Quantitative RT-PCR analysis showed that transcript of the strain 4B1 *dbfA1* gene was approximately 4-fold higher than that of strain YK5 after 1 and 3 h of cultivation with DF (Fig. 6). This result indicates that the *dbfA1* gene of strain 4B1 was highly expressed during early log phase, resulting in its rapid growth (Fig. 3). A nucleotide sequence alignment of the *dbf* genes showed that these two *Paenibacillus* strains have differences in their putative promoter regions (Fig. S1). Further analyses will be required to show whether these differences influence the transcription of these *dbf* genes.

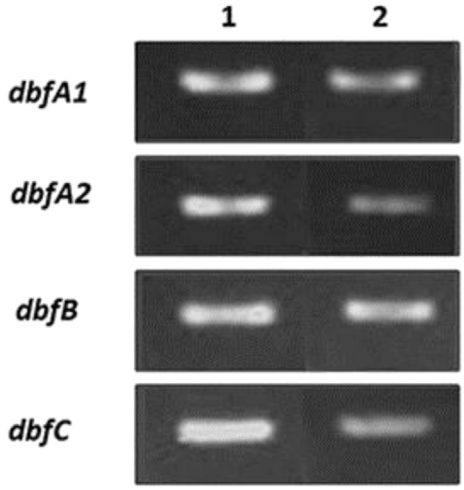

FIG. 5. RT-PCR analysis of *dbfA1A2BC* genes from *P. naphthalenovorans* strain 4B1 under induction of DF. cDNAs were generated from 2 μg of RNA samples extracted from cells grown on DF (lane 1) or succinate (lane 2).

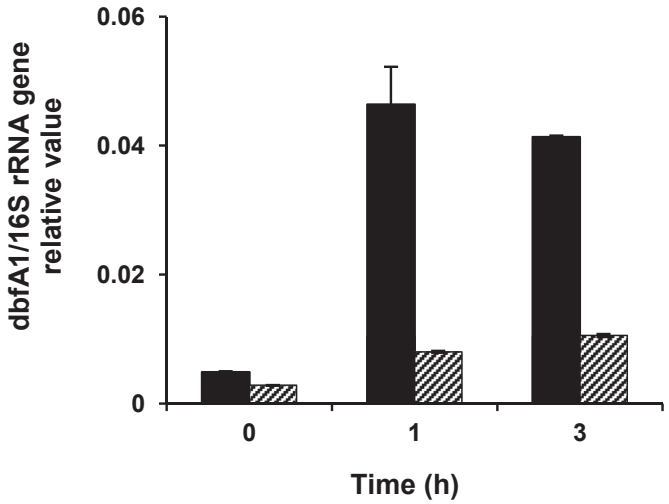

FIG. 6. Quantitative RT-PCR analysis of *dbfA1* gene of *P. naphthalenovorans* strain 4B1 (closed bars) and YK5 (hatched bars) grown in W medium containing 0.1% DF. 16S rRNA gene was used as the internal standard for normalizing the RNA samples. The data are means and standard deviations (error bar) of triplicates.

This study has demonstrated the isolation of a moderate thermophilic DF-utilizing bacterium, *P. naphthalenovorans* strain 4B1, from dioxin-contaminated soil in Vietnam. Strain 4B1 showed faster growth rate and higher DF degradation efficiency than the previously isolated DF-degrading *Paenibacillus* sp. strain YK5 under thermophilic condition. Although the *dbf* gene clusters involved in DF degradation in both strains were highly conserved, higher amounts of transcripts were detected in strain 4B1 in the presence of DF at 45°C. This was probably due to differences in the transcription regulation systems of the *dbf* genes in each strain. Further study in molecular levels would provide us the mechanism how the strain 4B1 could degrade DF more efficiently.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.05.006.

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