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A Pseudomonas sp. strain uniquely degrades PAHs and heterocyclic derivatives via lateral dioxygenation pathways

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(Article begins on next page)

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1	A Pseudomonas sp. strain uniquely degrades PAHs and heterocyclic	
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15		

16 Abstract

Polycyclic aromatic hydrocarbons (PAHs) and heterocyclic derivatives are organic pollutants 17 that pose a serious health risk to human beings. In this study, a newly isolated *Pseudomonas* 18 brassicacearum strain MPDS could effectively degrade PAHs and heterocyclic derivatives, 19 including naphthalene, fluorene, dibenzofuran (DBF) and dibenzothiophene (DBT). Notably, 20 strain MPDS is able to degrade fluorene. DBF and DBT uniquely via a lateral dioxygenation 21 pathway, while most reported strains degrade fluorene, DBF and DBT via an angular 22 dioxygenation pathway or co-metabolize them via a lateral dioxygenation pathway. Strain 23 MPDS completely degraded 50 mg naphthalene (in 50 mL medium) in 84 h, and OD₆₀₀ reached 24 1.0-1.1; while, it stabilized at OD₆₀₀ 0.5-0.6 with 5 mg fluorene or DBF or DBT. Meanwhile, 25 65.7% DBF and 32.1% DBT were degraded in 96 h, and 40.3% fluorene was degraded in 72 h, 26 respectively. Through genomic and transcriptomic analyses, and comparative genomic analysis 27 with another DBF degradation strain, relevant gene clusters were predicted, and a 28 naphthalene-degrading gene cluster was identified. This study provides understanding of 29 degradation of PAHs and their heterocyclic derivatives, as well as new insights into the lateral 30 dioxygenation pathway of relevant contaminants. 31

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Keywords: PAHs Degradation; Heterocyclic Derivative; *Pseudomonas*; Lateral Dioxygenation
Pathway

35

36 **1. Introduction**

Polycyclic aromatic hydrocarbons (PAHs) are organic pollutants that are composed of two or 37 more benzene rings (Haritash & Kaushik, 2009), such as naphthalene and fluorene. Heterocyclic 38 39 aromatic hydrocarbons are compounds with other elements composing the ring structures like dibenzofuran (DBF) and dibenzothiophene (DBT). The main anthropogenic sources of PAHs 40 and heterocyclic derivatives are from incomplete combustion of fossil and solid biomass fuels, 41 42 high-temperature industrial processes and petroleum refinery effluents (Sakshi et al., 2019). About 116,000 tons of PAHs have been produced since 2003 (Xu et al., 2006; Zhang et al., 43 2008), causing environmental pollution and threatening human health. Fluorene is a tricyclic 44 45 aromatic hydrocarbon with a strong toxic effect, and its heterocyclic derivatives (DBF and DBT) pose a serious health risk to human beings, resulting in deformities, cancer, gene mutation and 46 chromosome aberration through breathing or direct skin contact (Sakshi & Haritash, 2020). 47

48 Considering physical and chemical treatments of PAHs are energy, cost, chemical intensive, and even causing secondary pollutants, bioremediation is ecofriendly and sustainable, and has 49 recently gained considerable attention in the last two decades (Sakshi et al., 2019). Several 50 51 microorganisms that could utilize fluorene, DBF or DBT were screened and discovered, including Pseudomonas (Fortnagel et al., 1990; Grifoll et al., 1995; Li et al., 2009), 52 Sphingomonas (Wilkes et al., 1996; Gai et al., 2007), Rhodococcus (Alv et al., 2008), 53 Burkholderia (Gregorio et al., 2004), Rhizobium meliloti (Frassinetti et al., 1998), and 54 Terrabacter (Schmid et al., 1997; Kasuga et al., 2013). However, these reported strains only 55

utilize one of those pollutants as the sole carbon, or degrade DBF or DBT by co-metabolism
with other compounds. For example, Becher (2000) and Li (2009) respectively found *Ralstonia*sp. strain SBUG 290 and *P. putida* B6-2, which could co-metabolize DBF in cultivation with
biphenyl. *Arthrobacter* sp. P1-1 capable of co-metabolizing DBT with phenanthrene was
identified (Seo et al., 2006). These strains lack the ability to utilize DBF or DBT independently,
and few strains could degrade these three substrates simultaneously.

Through technologies of genomics, proteomics, transcriptomics and metabolomics, 62 researchers analyzed the microbial degradation pathways and genetic information of PAHs 63 (Sakshi & Haritash, 2020). Diverse clusters of PAH-catabolic genes such as nah, pah, nid, dox, 64 phn, phd, nag, fln were discovered in past research studies and the degradation pathways of 65 PAHs have been given more attention (Sakshi & Haritash, 2020). In recent years, various 66 degradation pathways of fluorene, DBF and DBT have been studied. There are two kinds of 67 biodegradation pathways for these three compounds: an angular dioxygenation pathway and a 68 lateral dioxygenation pathway. As for angular dioxygenation pathway, fluorene is transformed 69 into 9-fluorenol, 9-fluorenone and 1,9a-dihydroxy-1-hydrofluoren-9-one step by step (Trenz et 70 al., 1994; Nojiri et al., 2002; Nojiri et al., 2001; Wattiau, et al., 2001). Then, after several 71 uncertain steps, it is further catabolized to phthalic acid, which can be fully biodegraded by 72 many microorganisms. Luc Schuler found that genes *flnA* and *flnB* are responsible for the 73 angular oxidation of fluorene, 9-fluorenol and 9-fluorenone (Schuler et al., 2008). Another 74 degradation pathway of Arthrobacter sp. F101 proposed by Casellas et al. (1997) starts with the 75

lateral dioxygenation at C-3.4. After two-step oxidations and oxidative decarboxylation, it could 76 be transformed into 2-hydroxycinnamic acid. However, the related genes in this pathway have 77 rarely been reported. Lateral dioxygenation degradation pathways of DBF and DBT are quite 78 similar to that of fluorene. Li (2009) reported that the biphenyl gene cluster of P. putida strain 79 B6-2 generated HOBB (3'-Oxo-3'H-benzofuran-2'-vliden)but-2-enoic acid) by DBF lateral 80 dioxygenation degradation pathway: DBT could catalvzed be into 81 1,2-dihydroxy-dibenzothiophene, 4-[2-(3hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid and 82 3-hydroxy-2-formyl-phenylthiophene. However, the genes and functional enzymes related to 83 this degradation pathway were rarely reported. 84

85 In addition to the lateral dioxygenation degradation pathway, DBF and DBT could also be degraded in the angular dioxygenation pathway, with the attack of C-4 and C-4a at the first step. 86 Compared with the lateral dioxygenation degradation pathway, related genes in the angular 87 dioxygenation pathway were widely studied in recent years (Ji et al., 2017; Shi et al., 2013). 88 Genes *dbfA* and *dfdA* encode dibenzofuran 4.4a dioxygenase that transforms DBF into 89 cis-4,4a-dihydroxy-4,4a dihydrodibenzofuran (Sukda et al., 2009). Genes dbfb and dbfc covert 90 cis-4,4a-dihydroxy-4,4a dihydrodibenzofuran into salicylic acid. Moreover, genes dszABCD are 91 significant in DBT angular dioxygenation degradation (Ji et al., 2017; Gregorio et al., 2004; 92 Oldfield et al., 1997), which are involved in the transformation from DBT to dibenzothiophene 93 sulfoxide 2',3'-dihydroxybiphenyl-2-sulfinate, anthranilic acid and 2-hydroxypenta-2,4-dienoic 94 95 acid. Although several key genes and enzymes of DBF and DBT degradation have been reported, 96 current knowledge about the fluorene, DBF and DBT complete catabolic pathways and related97 degradation genes are still lacking.

In this study, we isolated *P. brassicacearum* MPDS, a high-efficiency degrading strain that 98 can utilize naphthalene, fluorene, DBF or DBT as the only carbon source. We inferred that strain 99 MPDS degraded fluorene, DBF and DBT by lateral dioxygenation pathways on the basis of 100 LC-MS results, which are unique compared with previous reports. We analyzed the genome and 101 transcriptome of strain MPDS and found a naphthalene-degrading gene cluster named as 102 nahAFBCED. Comparative genomic analysis with strain MPDS and another DBF degrader (P. 103 strain FA-HZ1) revealed potential DBF degradation genes (Ali et al., 2019). In summary, our 104 findings provide a further understanding of molecular mechanisms during the biodegradation of 105 naphthalene, fluorene, DBF and DBT, and will benefit bioremediation of contaminated 106 environments. 107

108

109 2. Experimental procedures

110 2.1. Chemicals and culture media

111 Naphthalene (\geq 99%, purity), dibenzofuran (DBF) and dibenzothiophene (DBT) (\geq 99%, purity)

112 were purchased from J&K Scientific Technology Co., Ltd. Fluorene (≥ 99%, purity) was

- 113 purchased from Aladdin Bio-Chem Technology Co., Ltd. Cells were cultured with LB medium
- and MSM medium (Lu et al., 2019). MH medium was used to detect antibiotic resistance.
- 115 Preparation method of MH medium (/L): starch 1.5 g, acid hydrolyzed casein 17.5 g and beef

116 paste 2.0 g.

117

118 2.2. Strain isolation and identification

Pseudomonas sp. strain MPDS was isolated from the soil and mud contaminated by PAHs and petrochemicals in Tianjin (China). The soil sample was added into 50 mL MSM medium containing 0.1% yeast extract and 20 mg naphthalene. After cultivating for 7 days, a total of 1 mL mixture was added into a new flask with fresh MSM medium containing 20 mg naphthalene and cultured for 7 days. After three enrichments, a single colony was isolated on MSM plate without yeast extract. The examination of the substrates revealed that strain MPDS could utilize fluorene, DBF and DBT for growth.

The DNA of strain MPDS extracted and primer 27F 126 was (5'-AGAGTTTGATCCTGGCTCA-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') were 127 used to sequence the whole region of 16S rRNA. The 16S rRNA sequence was alignment on 128 BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The software MEGA 5.2.2 was used to build a 129 phylogenetic tree of strain MPDS and strains with high 16S rRNA homologous by 130 neighbor-joining (NJ). 131

- 132
- 133 2.3. Antibiotic resistance detection of strain MPDS

134 *Pseudomonas* sp. MPDS grew to the exponential phase in 5 mL sterilized LB medium and 135 measured OD_{600} with UV spectrophotometer. The OD_{600} of bacterial solution was regulated as

7

the same as 0.5 M standard turbid solution. The bacterial solution was coated on MH medium
with an antibiotic table by sterile absorbent cotton at 30°C for one day. The diameter of the
bacteriostatic circle was measured and then resistance was judged. Preparation method of 0.5 M
standard turbid solution: 0.0048 mol/L BaCl₂ 0.5 mL, 0.18 mol/L H₂SO₄ 99.5 mL.

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141 2.4. Optimal cultivation and degradation conditions of PAHs and heterocyclic derivatives

To obtain the best culture and degrading conditions, various substrates, temperatures, revolving 142 speeds and substrate additions were performed for detection. Strain MPDS grew to the 143 exponential phase in sterilized MSM medium (50 mL) with different substrates at 30°C and 144 200 rpm; the precultures of four substrates were used as seed broths (each of the seed broths was 145 only cultured with one substrate). 4% (vol/vol) of seed broths were transferred into 50 mL MSM 146 medium containing naphthalene (50 mg) or fluorene (2.5 mg) or DBF (2.5 mg) or DBT (2.5 mg) 147 at various temperatures (25, 30, 37, and 42°C) with 200 rpm. To explore the appropriate 148 revolving speed, the seed broths were transferred into MSM medium at 50, 100 and 200 rpm at 149 an optimum temperature. In order to optimize the initial addition, the activated seed solution 150 was transferred into 50 mL sterilized MSM with different initial concentrations (naphthalene: 151 0.2, 1, and 2 mg/mL; fluorene or DBF or DBT: 0.05, 0.1, 0.2, 0.4, and 1 mg/mL) at 200 rpm and 152 25°C. The detection of the four substrates was by HPLC and parameters were consulted from 153 Lu's report (Lu et al., 2019), the detection wavelengths are 275 nm (naphthalene), 280 nm 154 (DBF), 254 nm (fluorene and DBT). The standard curves were fitted by using OriginPro 9.0 155

156 software (Fig. S2).

157

2.5. Determination of intermediates in biodegradation of naphthalene, fluorene, DBF and DBT 158 Strain MPDS grew into the exponential phase in LB medium at optimal conditions. After 159 collected, washed and resuspended, the cells without any substrate were starved at 30°C for 160 3 hours and then diluted with PBS buffer ($OD_{600} = 5.0$). Four substrates were respectively added 161 into resting cell (50 mL). After ethyl acetate (50 mL) extraction, the supernatant was 162 concentrated by rotary evaporation and dissolved with 1 mL ethyl acetate, then LC-MS was used 163 to detect samples at different reaction times. The mixture of 30 µL condensed extraction and 30 164 µL BSTFA (Bis(trimethylsilyl)trifluoroacetamide, a silvlation derivatization reagent) was placed 165 at 70°C for half an hour and detected by GC-MS (Liu et al., 2020). The LC-MS system (Agilent 166 6850/5975C) was as followings: the temperature of column was 30°C; flow rate was 0.4 167 mL/min; water: methanol = 20% : 80%; injection volume was 5 µL; scanning range was 190 -168 600 nm; detection wavelengths were 275 nm (naphthalene), 280 nm (DBF), 254 nm (fluorene 169 and DBT). 170

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172 2.6. Detection of HOBB degradation by resting cells of strain MPDS

A total of 0.2 mM HOBB (Liu et al., 2018) was added into resting cells of strain MPDS. The colors of the supernatant between the experimental group and control group (PBS buffer with 0.2mM HOBB) were observed (Fig. S3C). The standard curve of HOBB (Fig. S3A) and the HOBB residual at different time (Fig. S3B) was determined by HPLC (Liu et al., 2018).

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178 2.7. Genome sequencing and analysis

The genomic DNA was extracted by Wizard genomic DNA purification kit (Promega). Illumina 179 MiSeq and PacBio sequencing platforms were used to sequence the whole genome of strain 180 MPDS (Shanghai Personalbio Technology Co., Ltd), AdapterRemoval (Schubert et al., 2016) 181 and SOAPec (Luo et al., 2012) were used to remove the joint contamination and filter the 182 low-quality reads, respectively. We used HGAP (Chin et al., 2016) and CANU (Koren et al., 183 2017) software to assemble the data from PacBio sequencing platform to get the contig sequence. 184 The second generation of high-quality data was used to correct the third generation contig 185 results by Pilon (Walker et al., 2014). GeneMarkS (Besemer et al., 2001) 186 (http://exon.gatech.edu/GeneMark) software was used to predict protein-coding genes. 187 tRNAscan-SE (Lowe & Eddy, 1997) was used to predict tRNA, Barrnap software (0.9-dev) to 188 predict rRNA, and Rfam database (Kalvari et al., 2018) to predict other non-coding RNAs. The 189 coding-protein genes were annotated with NR (NonRedundant Protein Sequence Database), 190 KEGG (Kyoto Encyclopedia of Genes and Genomes), eggNog (Non-supervised Orthologous 191 Groups) databases and Swiss-Prot. 192

193

194 *2.8. Transcriptomic analysis*

195 Strain MPDS was cultured in glycerol medium (MSM+ 1% glycerol) and DBF medium (MSM

+ 5 mg DBF), respectively. The RNA was sequenced by on Illumina sequencing platform using 196 Next-Generation Sequencing (NGS) (Shanghai Personalbio Technology Co., Ltd). Cuttadapt 197 utilized 3' joints and filter low quality reads. Bowtie2 was to remove 198 (http://bowtie-bio.sourceforge.net/index.shtml) compared the filtered reads to the reference 199 (strain **MPDS** genome). Then Htseq 0.6.1p2 200 genome we used (http://www-huber.embl.de/users/anders/HTSeq) and DESeq (version 1.18.0) to count gene 201 expression and analyze differentially expressed genes, respectively. 202

203

204 2.9. RT-qPCR analysis

The RNAprep pure cell/bacteria kit (TianGen, China) was used to extract total RNA. The cDNA product was obtained after DNase I (Thermo Scientific) treatment and reversed transcription with Hifair II 1st Strand cDNA Synthesis Kit (Yeasen). RT-qPCR of six genes in naphthalene gene cluster and 16S gene (reference gene) was detected by quantitative PCR apparatus (qTOWER³G) with Hieff qPCR Green Master Mix (Yeasen) and fold change in gene expression was calculated by $2^{-\Delta\Delta Ct}$ (Lu et al., 2019). The program primers of each genes are shown in Table S3.

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213 2.10. Accession number(s)

214 *Pseudomonas* sp. MPDS was stored in China Center for Type Culture Collection (CCTCC)
215 under accession number M 2020186. The whole-genome sequence of strain MPDS was

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deposited into the NCBI database under the accession number CP054128.

217

218 *3*. **Results**

219 *3.1. Isolation and identification of strain MPDS*

A strain named MPDS efficient in degrading PAHs and heterocyclic derivatives was isolated, including naphthalene, fluorene, dibenzofuran and dibenzothiophene. The 16S rRNA gene of strain MPDS has 99% similarity sequence identity with *Pseudomonas brassicacearum*; the phylogenic tree reveals that strain MPDS is also closed to *P. brassicacearum* (Fig. 1). This identified strain MPDS has resistance to multiple antibiotics including chloramphenicol, ampicillin, and kanamycin (Table 1).

226

3.2. Growth conditions and degradation abilities with naphthalene, DBF, DBT and fluorene

To confirm the growth conditions and the degradation abilities of four substrates, the aerobic 228 strain MPDS was cultured with different revolving speeds, at different temperatures and 229 different amounts of naphthalene, fluorene, DBF and DBT. Strain MPDS hardly grew at high 230 temperatures (\geq 37°C) and the optimal growth temperature was 25°C. The growth of strain MPDS 231 with naphthalene as a substrate at 25°C was much better than at other temperatures (Fig. S1A) 232 while growth with fluorene, DBF or DBT at 25°C was just slightly better than that at 30°C (Fig. 233 S1B-D). The optimal growth revolving speed of strain MPDS was 200 rpm (Fig. S1E-H). 234 Because strain MPDS had a stronger capacity to degrade naphthalene, the optimal naphthalene 235

amount was much higher than those three substrates. When 50 mg naphthalene was added, strain 236 MPDS grew into the logarithmic phase faster than with 100 mg, and the OD_{600} of these two 237 amounts was basically stable at 1.0; thus, 50 mg was selected as the optimal amount of 238 naphthalene (Fig. S1I). For fluorene, DBF and DBT, although the growth of strain MPDS was 239 not affected by substrate addition (Fig. S1J-L), it grew better under the condition of 5 mg 240 substrates than other amounts. The optimal temperature and flask shaking speed of growth and 241 degradation were 25°C (Fig. S1A-D) and 200 rpm (Fig. S1E-H), respectively. The optimal 242 amount of naphthalene was 50 mg, while 5 mg was a suitable addition amount of fluorene or 243 DBF or DBT. 244

At the optimal conditions, the degradation ability of strain MPDS was tested. Strain MPDS could completely degrade 50 mg naphthalene in 84 h (Fig. 2A). A total of 65.7% DBF (5 mg in 50 mL) and 32.1% DBT (5 mg in 50 mL) could be degraded in 96 h and 40.3% fluorene (5 mg in 50 mL) could be degraded in 72 h (Fig. 2B-D).

249

250 *3.3. Genomic analysis of strain MPDS*

The whole genome of strain MPDS has a single circular chromosome (Fig. 3A), which contained 6,213,959 bases with the G + C content of 60.25%. Besides 5,534 protein-coding sequences, the circular chromosome contains five rRNA operons and 65 tRNA genes with the coding percentage of 87.3%.

255 The genes encoding the degradation of PAHs and heterocyclic aromatic hydrocarbons were

predicted on a 27,933 kbp region (Table S1). This region includes the necessary genes 256 responsible for naphthalene degradation. The enzyme encoded by *nahAaAbAcAd* is naphthalene 257 dioxygenase, generating cis-naphthalene dihydrodiol from naphthalene. NahA (NahAa, NahAb, 258 NahAc, NahAd) greatly resembles their homologs in *Pseudomonas stutzeri* (sharing > 98%) 259 amino acid identity) (Bosch et al., 1999). A putative transcriptional regulator from AraC family 260 was located upstream of *nahAa*, with 99.0% amino acid homology with the homologues in 261 Pseudomonas sp. A214. The putative genes nahBFCED were involved in the conversion of 262 cis-naphthalene dihydrodiol to salicylic acid (Fig. 3B), which showed high identity to their 263 homologs in P. stutzeri (> 99% amino acid sequence identity) (Bosch et al., 1999) and the 264 predicted nahHIJKL (Yen & Gunsalus, 1985) are genes that biodegrade salicylic acid and 265 catechol, which have at least 98% amino acid sequence homology. 266

Based on genome annotation, other aromatic hydrocarbon degradation genes in this strain were also found including 4-hydroxybenzoate 3-monooxygenase, 4-hydroxyphenylacetate isomerase, and 3-phenylpropionate dioxygenase (*hcaE*). The substrates of these enzymes are mono benzene ring compounds; thus, it is speculated that they are involved in the degradation of downstream metabolites in the process of degrading PAHs. We also predicted some functional genes that are predicted to be essential for utilizing nitrate, synthesizing amino acids, transferring and absorbing C-containing compounds.

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275 3.4. Clarification of biodegadation pathways of naphthalene, DBF, DBT and fluorene

276 *3.4.1.* The naphthalene degrading pathway

The intermediates produced during naphthalene degradation were identified by LC-MS. 1,2-dihydroxynaphthalene, salicylic acid and catechol appeared at 4.200 min, 3.152 min, and 3.834 min, respectively, and the data of m/z by the mass spectra were 159.0456 (1,2-dihydroxynaphthalene), 137.0247 (salicylic acid) and 109.0304 (catechol), respectively (Fig. 4A). Thus, we speculated that the naphthalene degradation pathway was a salicylic acid pathway (Fig. 4B). This was consistent with the degradation pathway driven by naphthalene degradation gene clusters.

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285 *3.4.2. The DBF degrading pathway*

From the fermentation samples of DBF, new intermediate peaks were detected and identified, 286 including 3-(3'-oxobenzofuran-2'-vl)propanoic acid, 2-(3'-oxobenzofuran-2'-vl) acetic acid; 287 2-(3'-hydroxy-2',3'-dihydrobenzofuran-2'-yl) acetic acid, 2-oxo-2-(2-hydroxyphenyl) acetic 288 acid; 2-hydroxy-2-(2-hydroxyphenyl) acetic acid; salicylic acid; and catechol (Fig. 4F). The 289 intermediates appeared at 4.570 min, 3.293 min, 3.742 min, 2.878 min, 2.928 min, 3.859 min 290 and the data of m/z by mass spectra are shown in Fig. 4F. However, the most pivotal metabolite 291 HOBB (2-hydroxy-4-(3'-oxo-3'H-benzofuran-2'-yliden) but-2-enoic acid), a yellow toxic 292 intermediate metabolite produced during the lateral dioxygenation degradation pathway of DBF, 293 was not identified. We then prepared HOBB (Liu et al., 2018) as the only substrate for a resting 294 cell reaction and the results showed that strain MPDS degraded 0.2 mM HOBB in 45 h (Fig. 295

S3B-C) while many reported DBF-degrading strains had no ability to degrade HOBB efficiently and rapidly. This suggested that strain MPDS could biodegrade DBF via the lateral dioxygenation degradation pathway and would solve the problem in which this pathway is generally interrupted by HOBB. At the same time, we used GC-MS to detect silylated salicylic acid (after derivatization) at 11.097 min (Fig. 4G). The results illustrated that strain MPDS possesses the lateral dioxygenation pathway of DBF with the first step of hydrolysing DBF to HOBB and then degrading into salicylic acid (Fig. 4H).

- 303
- 304 *3.4.3.* The DBT degrading pathway

For DBT. typical products in the DBT lateral degradation pathway, 305 1,2-dihydroxydibenzothiophene, 3-hydroxy-2-formyl benzothiophene, 306 2.3-dihydroxybenzothiophene and thiosalicylic acid, were detected by LC-MS (Fig. 4I). These 307 intermediates appeared at 18.513 min, 3.018 min, 3.800 min and 2.918 min, respectively, and 308 the datas of m/z by mass spectra were 215.0176 (1,2-dihydroxydibenzothiophene), 177.0024 309 (3-hydroxy-2-formyl benzothiophene), 165.0065 (2,3-dihydroxybenzothiophene) and 153.0013 310 (thiosalicylic acid), respectively (Fig. 4I). Simultaneously, silvlated thiosalicylic acid was 311 detected by GC-MS (after derivatization) at 12,692 min (Fig. 4J). These results suggested that 312 strain MPDS degraded DBT into thiosalicylic acid by the lateral dioxygenation pathway (Fig. 313 4K). 314

315

316 *3.4.4. The fluorene degrading pathway*

9-Fluorenol, 2-propanoic acid-1-indanone and 2-acetic acid-1-indanone were identified by 317 LC-MS from fluorene samples (Fig. 4C) at 7.179 min, 3.217 min and 3.217 min, respectively. 318 Moreover, we detected related metabolites of 9-fluorenone by GC-MS (after derivatization) at 319 12.951 min (Fig. 4D). 9-Fluorenol and 9-fluorenone are metabolites in the angular 320 dioxygenation pathway: 2-propanoic acid-1-indanone and 2-acetic acid-1-indanone belong to 321 the lateral dioxygenation pathway. Thus, we inferred that strain MPDS might contain both 322 angular and lateral dioxygenation pathways (Fig. 4E) but the metabolic pathway of fluorene 323 degradation remains to be further studied. 324

In summary, biodegradation of DBF, DBT and fluorene is via the lateral dioxygenation pathways in strain MPDS, which is usually found in co-metabolism strains. We did not find similar reported genes for degradation of DBF, DBT or fluorene in the genome of strain MPDS. Therefore, we inferred that strain MPDS may carry new metabolic gene clusters of the lateral dioxygenation pathways for DBF, DBT and fluorene degradation.

330

331 *3.5. Transcriptional analysis*

In order to search for potential degradation gene clusters, we performed transcriptome analysis. In view of physiological and biochemical properties, strain MPDS has a stronger ability to degrade DBF than DBT or fluorene. Therefore, we chose DBF as the substrate for transcriptome data analysis to search for functional genes. According to the transcriptome results, 870 genes show differential expression levels between the negative control group (cultured by 1% glycerol)
and experimental group (cultured by DBF). On the conditions of log2|fold change|>1 and
P-values <0.05, 487 genes were up-regulated and 383 genes were down-regulated (Fig. 5A and
D). Genes with similar expression levels were identified by cluster analysis (Fig. 5B). The
enrichment analysis of KEGG pathway found that differentially expressed genes were mainly
related to the degradation of valine, leucine and isoleucine, the metabolism of glyoxylate and
dicarboxylate, and the metabolic pathway of propanoate (Fig. 5C).

The initial genes encoding the enzyme (NahA) of the naphthalene degradation pathway 343 were up-regulated induced with DBF or its intermediates, and the gene encoding salicylaldehyde 344 dehydrogenase was also highly expressed. Moreover, quantities of other predicted genes might 345 play significant roles during DBF degradation were also up-regulated, such as the coding genes 346 of 4-hydroxybenzoate 3-monooxygenase, 4-hydroxyphenylacetate isomerase. 347 and 3-phenylpropionate dioxygenase (hcaE). We also found 107 potential and unknown-function 348 genes, which were up-regulated and might be essential to DBF degradation, were deserved 349 further attention. However, we did not find any genes sharing high similarity with the reported 350 DBF-degrading genes, which indicated that there would be novel DBF-degrading genes of 351 unknown function in those up-regulated gene clusters, such as the putative protein genes in the 352 vicinity of *nahAFBCED*, the upstream or downstream hypothetical protein genes of monophenyl 353 compound metabolism genes and other clusters of hypothetical protein genes with unknown 354 functions. 355

357 3.6. Comparative genomic analysis of DBF degradation strains

In a previous study, we isolated and reported on another DBF-degrading *Pseudomonas* sp. strain 358 FA-HZ1 (Ali et al., 2019). To obtain comprehensive information of the DBF-degrading pathway 359 in strain MPDS and to screen more potential functional genes, we analyzed the genomic 360 sequences of these two strains together. These two strains showed high sequence identity (more 361 than 80%) in 249 genes. Most of them are responsible for basic metabolism activities, including 362 DNA replication and repair, transcription regulatory factors, ribosomal protein synthesis, amino 363 acid degradation and transport, aldehyde dehydrogenase, carbohydrate metabolism enzymes and 364 so on. In addition to these genes with clear functions, we also found 13 genes with highly 365 similar sequences that were up-regulated in transcriptome data of strain MPDS (Table S2 and 366 Fig. 6). Particularly, we found that the expression of a hypothetical protein (gene 4284), which 367 was downstream of the naphthalene degradation gene cluster, was up-regulated by 7.8 times and 368 there were seven up-regulated genes nearby. 369

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371 *3.7. Real-time quantitative PCR (RT-qPCR) of naphthalene gene cluster*

We found a naphthalene-degrading gene cluster in strain MPDS, according to genomic data and transcriptome data growing with DBF, and the expression of *nahAa*, *nahAb* and *nahAc*, which encode naphthalene 1,2-dioxygenase, was up-regulated. To determine the role of this gene cluster in naphthalene degradation, we performed real-time quantitative PCR of 16S rRNA gene

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(reference gene) and six genes (nahA, nahB, nahD, nahF, nahH and nahO), which located 376 upstream or downstream of naphthalene degradation gene cluster (Fig. 7). The expression of 377 *nahA* and *nahB* that encoded naphthalene dioxygenase and dehydrogenase were highly 378 up-regulated by 37.8 times and 42.2 times compared with control group (glycerol). These two 379 genes are initial and pivotal in naphthalene degradation and degrade naphthalene into 380 1.2-dihydroxynaphthalene. Another high expression gene *nahH*, which encoded catechol 381 2,3-dioxygenase, was up-regulated by 36.1 times. However, other three genes (nahF, nahD, and 382 *nahO*) did not show up-regulated evidently, which could encode salicylaldehyde dehydrogenase, 383 2-hydroxychromene-2-carboxylate isomerase and acetaldehyde dehydrogenase. 384

The RT-qPCR results suggest that express of some genes in the naphthalene gene cluster of strain MPDS might be induced by naphthalene or its intermediate metabolites, but some of them were constitutive expression. The regulatory mechanism of naphthalene degradation in strain MPDS needs further exploration.

389 4. Discussion

There are many reports about degradation strains of naphthalene, fluorene, DBF and DBT. *Pseudomonas* (Bosch et al., 1999; Yen & Gunsalus, 1985; Ali et al., 2019), *Rhodococcus* (Andreoni et al., 2000), *Micrococcus* (Wen et al., 2006), *Alcaligenes* (Guerin & Boyd, 1995) and *Corynebacterium* can degrade naphthalene. Besides *Pseudomonas* and *Rhodococcus*, there are several microorganisms that can utilize fluorene, DBF or DBT, including *Sphingomonas* (Wilkes et al., 1996; Gai et al., 2007), *Burkholderia* (Gregorio et al., 2004), *Rhizobium meliloti*

(Frassinetti et al., 1998), Terrabacter (Schmid et al., 1997; Kasuga et al., 2013) and so on. Many 396 researchers have studied the angular dioxygenation pathway and lateral dioxygenation pathway 397 of fluorene, DBF or DBT and several pivotal genes in angular dioxygenation pathway have been 398 reported while genes in the lateral dioxygenation pathway remained to be discovered. The lateral 399 degradation pathway of DBF and DBT is mainly through co-metabolism. For example, Stope et 400 al. (2002) found that *Rhodococcus ervthropolis* SBUG 271 cultured with biphenvl could 401 co-metabolize DBF. Seo et al. (2006) isolated Arthrobacter sp. P1-1 that co-metabolized DBT 402 with phenanthrene. These strains are incapable of utilizing DBF or DBT independently, thus, 403 specific genes involved in this pathway have not been reported. On the other hand, theses 404 degradation pathways by co-metabolizing microorganisms are usually blocked at a certain step. 405 For example, DBF degradation is generally interrupted due to toxic product HOBB, while 406 DBT-degrading strains usually cannot fully desulfurize DBT, resulting in the accumulation of 407 3-hydroxy-2-formyl benzothiophene. Therefore, it is a significant advantage that strain MPDS 408 can degrade naphthalene, fluorene, DBF and DBT, which is rare in past studies. Moreover, the 409 reported genes have not been found in the strain MPDS genome, which suggests that strain 410 MPDS might carry novel functional genes of PAHs degradation, and deserves further study. 411

The degradation genes of naphthalene in *P. putida* G7, *P. putida* NICB9816 (Simon et al., 1993) and *P. stutzeri* AN10 (Bosch et al., 1999) were studied in detail. The degradation genes were found both on plasmids and chromosomes in different strains. For example, gene cluster *nahAaAbAcAdBFCED* of *P. stutzeri* AN10 are distributed on chromosomes, and the

naphthalene-degrading genes from P. putida G7 are on NAH7 plasmid. Notably, NAH7 plasmid 416 from P. putida G7 contains two different gene degradation pathway clusters. The first gene 417 cluster is *nahAaAbAcAdBFCED* that degrade naphthalene into salicylic acid and another gene 418 cluster *nahGHILJKMNOR* completely biodegraded via catechol. Besides the catechol pathway, 419 there is a gentianic acid pathway in naphthalene degradation. The beginning of another 420 degradation pathway is as the same as the first one, while the second part of the pathway is via 421 gentianic acid not catechol. The researchers also found other highly conserved 422 naphthalene-degrading gene clusters, such as NAH-like gene cluster, pah gene cluster of P. 423 putida ous82, nah gene cluster on pDTG1 from P. putida NICB9816-4 and dox gene cluster on 424 C18 plasmid of Pseudomonas C18 (Kiyohara et al., 1994; Dennis & Zylstra, 2004; Takizawa et 425 al., 1999). For strain MPDS, *nahAaAbAcAdBFCED* was identified clearly, and is highly similar 426 to the gene cluster of *P. stuttzeri* (sequence homology > 98%). In addition, we found a catechol 427 degradation gene near the naphthalene gene cluster in strain MPDS. Thus we suggest that the 428 degradation is via salicylic acid pathway. 429

To obtain a comprehensive information of the DBF-degrading pathway in strain MPDS and screen more potential functional genes, we analyzed the genomic sequences of strain MPDS along with strain FA-HZ1 (Ali et al., 2019). These two strains showed high sequence identity (more than 80%) in 249 genes and 13 of these genes were up-regulated in transcriptome data of strain MPDS (Table S2 and Fig. 6). Particularly, we found that the expression of a hypothetical protein (gene 4284), which was downstream of the naphthalene degradation gene cluster, was up-regulated by 7.8 times and there were seven up-regulated genes nearby. These unknowngenes might be related to the degradation of DBF and deserve further study.

In summary, the *Pseudomonas* strain MPDS is able to utilize naphthalene, fluorene, and 438 their heterocyclic derivatives DBF and DBT as the only carbon sources for growth, and could 439 biodegrade fluorene, DBF, and DBT via lateral dioxygenation metabolic pathways, which are 440 rare in previous studies. Considering the lack of molecular mechanisms and biochemical 441 properties of the lateral dioxygenation pathways of fluorene, DBF, and DBT, strain MPDS could 442 be a significant microbial resource in degrading PAHs. We determined the degradation ability of 443 strain MPDS, clarified the degradation pathways of four substrates, identified the functional 444 genes of naphthalene degradation, and analyzed genomic sequences and transcriptome data of 445 potential genes. In further research, we will exploit novel genes of the lateral dioxygenation 446 metabolic pathways of fluorene, DBF and DBT degradation, and give more information about 447 molecular mechanisms of lateral dioxygenation metabolic pathways. Such work will benefit the 448 bioremediation of sites contaminated by PAHs and heterocyclic aromatics. 449

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451 CRediT authorship contribution statement

452 YL liu, and HY Hu performed the experiments. HZ Tang and HY Hu designed the experiments.

453 YL liu, HY Hu and HZ Tang wrote the manuscript. P Xu, HZ Tang and Giulio Zanaroli revised

the manuscript. P Xu and HZ Tang conceived the project.

455 Declaration of Competing Interest

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456 None.

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458	Acknowledgments
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613 Figure legends

Fig. 1. Phylogenetic relationships of strain MPDS. Neighbor-Joining phylogenetic tree of 16S
rRNA sequences of strain MPDS, with 1,000 bootstrap replications, is shown. The accession
numbers in phylogenetic tree are from NCBI database and RDP database. The strains with "T"
are type bacteria. The phylogenetic tree revealed that strain MPDS is closed to *Pseudomonas brassicacearum*.

Fig. 2. Degradation abilities of strain MPDS cultured with naphthalene (A), fluorene (B), DBF
(C) and DBT (D) under optimal growth conditions. CK: MSM without strain MPDS. EG: MSM
with strain MPDS.

Fig. 3. Genomic analysis of strain MPDS. (A) Genome map. From inside to outside, circle 1 is 622 scale; circle 2 is G + C skew; circle 3 is the content of G + C; circles 4 and 7 are clusters of 623 orthologous groups; circles 5 and 6 are locations of coding sequence, tRNA, and rRNA genes. 624 (B) Predicted naphthalene degradation gene cluster in strain MPDS. Genes with different color 625 represent different functions (blue, naphthalene hydrolysis; yellow, salicylic acid degradation). 626 The sizes and directions of arrows indicate the sizes and transcription direction of genes. The 627 genes are summarized in Table S1. (C) Reported naphthalene degradation gene clusters. (Grav. 628 naphthalene-degradation gene cluster from plasmid NAH7 in Pseudomonas putida G7; orange, 629 dox gene cluster on C18 plasmid of *Pseudomonas* C18; pink, naphthalene-degradation gene 630 631 cluster from Pseudomonas stutzeri AN10).

632	Fig. 4. Pathways proposed for the degradation and identification of intermediates of naphthalene,
633	DBF, DBT and fluorene by strain MPDS. (A) LC-MS mass spectra of naphthalene derivatives:
634	1,2-dihydroxynaphthalene (II), salicylic acid (V), catechol VI). (B) Proposed dioxygenation
635	pathway of naphthalene. (C) LC-MS mass spectra of fluorene derivatives: 9-fluorenol (I),
636	2-Propanoic acid-1-indanone (IV) and 2-acetic acid-1-indanone (V). (D)GC-MS mass spectra of
637	fluorene derivative: 9-fluorenone (II). (E) Proposed lateral dioxygenation pathway of fluorene.
638	(F) LC-MS mass spectra of DBF derivatives: 3-(3'-oxobenzofuran-2'-yl)propanoic acid (III),
639	2-(3'-oxobenzofuran-2'-yl)acetic acid (IV), 2-(3'-hydroxy-2',3'-dihydrobenzofuran-2'-yl)acetic
640	acid (V), 2-oxo-2-(2-hydroxyphenyl)acetic acid (VI), salicylic acid (VII) and catechol (VIII).
641	(G)GC-MS mass spectra of DBF derivative: silylation salicylic acid (VIII). (H) Proposed lateral
642	dioxygenation pathway of DBF. (I) LC-MS mass spectra of DBT derivatives:
643	1,2-dihydroxydibenzothiophene (I), 3-hydroxy-2-formyl-benzothiophene (III),
644	2,3-dihydroxybenzothiophene (IV) and thiosalicylic acid (V). (J) GC-MS mass spectra of DBT
645	derivative: silanized thiosalicylic acid (V). (K) Proposed lateral dioxygenation pathway of DBT.

Fig. 5. Transcriptome analysis in strain MPDS induced by DBF. (A) The numbers of genes which were up-regulated or down-regulated induced with DBF. (B) Hierarchical cluster heat map of gene expression in strain MPDS. DBF, experimental group cultured with DBF; CK, control group cultured with glycerol. (C) The enrichment analysis of KEGG pathways of differentially expressed genes induced with DBF. (D) Volcano Plot. The abscissa is the value of log2 of fold change. The ordinate is the value of -log10 of p-value. In this Figure, the vertical line represents 2-fold change threshold; the horizontal line represents the p-value of 0.05. Every gene is represented by a point, red points represent up-regulated genes while blue points represent down-regulated genes, gray points represent nonsignificant differentially expressed genes.

Fig. 6. Up-regulated genes in strain MPDS induced with DBF that have high sequence identity(more than 80%) with strain FA-HZ1.

Fig. 7. RT-qPCR of genes related to degrading naphthalene in strain MPDS; 16S rRNA was
regarded as a reference gene. Different colors of bars represent different functional genes.
Experience group: 50 mg naphthalene induction; control group: 1% glycerol.



Fig. 1

Fig. 2







1Kb





Fig. 5







Pseudomonas sp. DBF1

Fig. 7



Antibiotic	Average diameter of bacteriostatic circle(cm)	Antibiotic resistance
Amikacin	2.680	S
Tetracycline	3.025	S
Kanamycin	2.375	S
Gentamicin	1.800	S
Piperacillin	3.450	S
Fosfomycin	3.625	S
Streptomycin	1.325	Ι
Rifampicin	1.925	Ι
Ciprofloxacin	1.725	Ι
Lincomycin	0.750	R
Amoxicillin	0.700	R
Norfloxacin	0.950	R
Chloramphenicol	0.700	R
Cefazolin	0.700	R
Nitrofurantoin	0.700	R
Penicillin	0.700	R
Ampicillin	0.700	R
Vancomycin	0.700	R
Erythromycin	0.700	R
Clindamycin	0.700	R

Table 1. Antibiotic resistance of strain MPDS

R: resistance, I: intermediary, S: sensitivity.