Characterization of a Novel Angular Dioxygenase from Fluorene-Degrading *Sphingomonas* sp. Strain LB126

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In this study, the genes involved in the initial attack on fluorene by *Sphingomonas* sp. strain LB126 were investigated. The α and β subunits of a dioxygenase complex (FlnA1-FlnA2), showing 63 and 51% sequence identity, respectively, to the subunits of an angular dioxygenase from the gram-positive dibenzofuran degrader *Terrabacter* sp. strain DBF63, were identified. When overexpressed in *Escherichia coli*, FlnA1-FlnA2 was responsible for the angular oxidation of fluorene, 9-hydroxyfluorene, 9-fluorenone, dibenzofuran, and dibenzo-p-dioxin. Moreover, FlnA1-FlnA2 was able to oxidize polycyclic aromatic hydrocarbons and heteroaromatics, some of which were not oxidized by the dioxygenase from *Terrabacter* sp. strain DBF63. The quantification of resulting oxidation products showed that fluorene and phenanthrene were the preferred substrates of FlnA1-FlnA2.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants and are released during the burning, handling, or disposal of organic matter including coal tars, crude oil, and petroleum products. There are some natural origins, such as forest fires and natural oil seeps, but PAHs arise mainly from combustion- or oil-related anthropogenic activities. A number of organisms that are able to use PAHs as the sole source of carbon and energy have been isolated previously (6), and bioremediation strategies using these organisms have been proposed (17).

Fluorene, a tricyclic aromatic hydrocarbon containing a five-membered ring, offers a variety of possibilities for biochemical attack. Two of these pathways are initiated by dioxygenation at the 1,2 (5, 9) or 3,4 (5, 10, 28) positions (Fig. 1). The corresponding cis-dihydrodiols undergo dehydrogenation and then meta cleavage. The third route (38, 44) is initiated by mono-oxygenation at the C-9 position to give 9-hydroxyfluorene, which is then dehydrogenated into 9-fluorenone. This route is productive only if subsequent angular carbon dioxygenation forms 1-hydro-1,1a-dihydroxy-9-fluorenone, leading to phthalate, which is further degraded via protocatechuate (11, 28, 44) (Fig. 1).

Sphingomonads have been intensively studied for their ability to degrade a wide range of aromatic hydrocarbons (32, 34, 41, 42, 48, 49). The function and organization of PAH catabolic genes in *Sphingomonas* species often remain obscure since the genes involved in the degradation of aromatic compounds are not always arranged in discrete operons but are frequently dispersed throughout the genome. *Sphingomonas* sp. strain LB126 was isolated from PAH-contaminated soil and is capable of utilizing fluorene as the sole carbon source (3). Fluorene degradation by strain LB126 has been investigated previously (47), but the enzymes that govern the initial attack on fluorene were not identified.

In contrast, more genetic work done with gram-positive bacteria (13, 14) has shown that dibenzofuran-degrading *Terrabacter* sp. strain DBF63 can also oxidize fluorene, thanks to a cluster of plasmid-borne catabolic genes. The oxygenase component of an angular dioxygenase complex, encoded by *dbfA1* and *dbfA2*, does not cluster with already known dioxygenases. Few data are available regarding genes involved in fluorene degradation by gram-negative bacteria. Although many PAH dioxygenases can oxidize fluorene, the respective host strains cannot use fluorene as the sole carbon source. Recently, the catabolic plasmid pCAR3 from *Sphingomonas* sp. strain KA1 was described (40). Genes homologous to *dbfA1* and *dbfA2*, as well as all genes necessary for the complete degradation of fluorene, were found on pCAR3, but strain KA1 is unable to grow on fluorene as the sole source of carbon. We present here the first report, to our knowledge, of genes governing the angular attack on fluorene by the gram-negative *Sphingomonas* sp. strain LB126 using fluorene as the sole source of carbon and energy.

MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** *Sphingomonas* sp. strain LB126 uses fluorene as the sole source of carbon and energy (3) and was kindly provided by Vlaamse Instelling voor Technologisch Onderzoek, Belgium. *Escherichia coli* Top10 was used as the recipient strain in all cloning experiments. *E. coli* BL21(DE3) was used for gene expression analysis. PCR amplicons were cloned into either pDrive (Qiagen, Valencia, CA), pGEM-T Easy vector (Promega,
charged nylon membrane (Amersham, Buckinghamshire, United Kingdom) by using standard protocols (36). For Southern blot detection, a PCR-amplified digoxigenin-labeled probe was prepared according to the recommendations of the manufacturer (Roche Diagnostics, Mannheim, Germany) by using primers described by Iida et al. (15). Prehybridization and hybridization were carried out at 68°C. After hybridization, the membrane was washed twice with 2× SSC (20× SSC is 3 mol of NaCl/liter and 0.3 mol of sodium citrate/liter, pH 7.0) containing 0.1% (wt/vol) sodium dodecyl sulfate (SDS) for 5 min at room temperature and twice with 0.1× SSC containing 0.1% SDS for 15 min at 68°C. Detection was carried out according to standard protocols (36). To isolate catabolic genes, total DNA (10 μg) was digested with BamHI and NsiI and separated by gel electrophoresis and DNA fragments of about 7 kb were recovered from the agarose gel by using the UltraClean GelSpin DNA extraction kit (MoBio, Carlsbad, CA). The DNA obtained was cloned into pGEMZ (Promega, Madison, WI), and E. coli Top10 cells (Invitrogen, Carlsbad, CA) were transformed with the resulting construct. Transformants were screened as pools of 10 clones by PCR using the above-mentioned primers (15). A 6.9 kb plasmid containing the angular dioxygenase gene was identified and sequenced by primer walking.

Construction of plasmids for protein overexpression. The construction of the plasmids used in this study involved multiple PCR amplifications and cloning steps. The flnA1-flnA2 fragment (1,842 bp) was amplified by PCR with the primer pair 5′-CATATGGCCACAGGCTCTAGAAACC-3′ and 5′-AACCTGG CGCTCACAGGAACACCG-3′, introducing Ndel and HindIII sites (indicated by italics) at the ends of the amplicon. The PCR product was cloned into pDrive (Qiagen), sequenced, and then subcloned into the Ndel and HindIII sites of expression vector ρET-308 (Novagen). E. coli BL21(DE3) was transformed with this construct for expression analysis.

SDS-PAGE. Bacterial cells were pelleted by centrifugation and washed with 10 ml of ice-cold phosphate buffer (140 mM NaCl, 10 mM Na2HPO4, 2.7 mM KCl, pH 7.2) instead of Tris buffer. Fluorene was provided as crystals in both solid and liquid phases, which was incubated at 42°C up to an OD600 of 0.5. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.5 mM. The cells were further incubated for 7 h at 25°C. For in vivo assays, cells were centrifuged, washed, and resuspended to an OD600 of approximately 2 in M9 medium (36) containing 0.2% glucose. Cells (25 ml) were incubated for 48 h at 25°C with 5 ml of silicone oil containing each tested PAH at 0.1 gliter.

GC-MS analysis of PAH oxidation products. Water-soluble products resulting from PAH oxidation were extracted from the aqueous phase of the bacterial suspensions by using columns filled with reverse phase-bonded silica (Chrompack, C18, 5 μm; Interchem, Montluçon, France). Columns were washed with 10 ml of water and then eluted with 1 ml of ethyl acetate. The solvent was dried over sodium sulfate and evaporated under nitrogen gas. The dried extracts were then dissolved in 100 or 200 μl of acetonitrile before being derivatized with N,N-diethyl trifluoroaceticamide:trimethylchlorosilane (BSTFA) or α-n-butyl-borionate (NBB). In order to quantify the dihydrodiols formed upon the incubation of BL21(DE3)pET30flnA1A2 recombinant cells with PAHs, 2,3-dihydrodiphenyl (Sigma-Aldrich, St. Louis, MO) was added to a 0.1 μM final concentration in the aqueous phase prior to solid-phase extraction and was used as an internal standard. After derivatization and gas chromatography (GC)-mass spectrometry (MS) analysis, NBB dihydrodiol derivatives were quantified on the basis of peak areas by using a calibration curve generated by analyzing known amounts of anthracene 1,2-dihydrol on (trimethylsilyl) trifluoroaceticamide:trimethylchlorosilane (BSTFA) or α-n-butyl-borionate (NBB). In order to quantify the dihydrodiols formed upon the incubation of BL21(DE3)pET30flnA1A2 recombinant cells with PAHs, 2,3-dihydrobiphenyl (Sigma-Aldrich, St. Louis, MO) was added to a 0.1 μM final concentration in the aqueous phase prior to solid-phase extraction and was used as an internal standard. After derivatization and gas chromatography (GC)-mass spectrometry (MS) analysis, NBB dihydrodiol derivatives were quantified.

FIG. 1. Proposed pathways for fluorene degradation and the bacteria involved. 1, Anthrobacter sp. strain F101 (5, 9); 2, Terrabacter sp. strain DBF63 (28); 3, Brevibacterium sp. strain DPO1361 (44); 4, Pseudomonas sp. strain F274 (11); 5, Burkholderia cepacia F297 (12); and 6, Sphingomonas sp. strain LB126 (47).
then used as a digoxigenin-labeled probe in Southern blot experiments with whole-genome extracts of strain LB126. A 6.9-kb fragment encompassing four open reading frames (ORFs; orf3 to orf6) and three truncated ones (orf1, orf2, and orf7) was cloned and sequenced as described in Materials and Methods (Table 1). The orf1 gene product did not show amino acid sequence similarities to previously described fluorene catabolic gene products but showed significant homology to the TonB-dependent receptor CirA from Sphingomonas wittichii strain RW1 (36%) and Novosphingobium aromaticivorans F199 (34%). orf2 encoded a truncated transposase, suggesting that the adjacent gene cluster was probably acquired by horizontal transfer, although no change in G + C content was noticed. orf3 to orf7 showed genetic organization similar to that of the dibenzofuran catabolic operon from Terrabacter sp. strain DBF63 (20) (Fig. 2). Nevertheless, the product of orf3, a putative dehydrogenase, did not show significant protein sequence similarity to its counterpart (FlnB) from strain DBF63. The amino acid sequences of the subunits showed moderate identity (63 and 51%) to the sequences of DbfA1 and DbfA2 from strain DBF63. Phylogenetic analysis revealed that the orf4 product did not cluster with dioxygenase α subunits from other sphingomonads and was only distantly related to the angular dioxygenase from S. wittichii strain RW1 (4). The closest homologues within sphingomonads were the dioxygenase α subunits from the carba-zole-degrading strains Sphingomonas sp. strain KA1 (36%)}

**RESULTS AND DISCUSSION**

Cloning and sequence analysis of genes encoding a novel angular dioxygenase. *Sphingomonas* strain LB126 has been studied for its ability to grow on fluorene and degrade phenanthrene, anthracene, and fluoranthene by cometabolism in the presence of pyruvate (46). In order to detect genes potentially involved in the initial attack of PAHs, a PCR strategy was chosen. The genes in strain LB126 involved in fluorene oxidation were expected to display some similarity to previously described counterparts in other PAH-degrading *Sphingomonas* strains. Several primer pairs corresponding to conserved domains of previously described PAH dioxygenases were tested (7, 19, 24, 29), but no amplification could be obtained (data not shown). Given the dearth of information regarding fluorene degradation genes in gram-negative bacteria, primers specific to angular dioxygenase genes from gram-positive bacteria were chosen. The genes in strain LB126 involved in fluorene oxidation (see below) showed genetic organization similar to that of the dibenzofuran catabolic operon from Terrabacter sp. strain DBF63 (20). Nevertheless, the product of orf3, a putative dehydrogenase, did not show significant protein sequence similarity to its counterpart (FlnB) from strain DBF63. The highest degree of similarity was found with putative dehydrogenases from *Mycobacterium* sp. strain MCS (orf4) and *Mycobacterium* sp. strain KMS (orf5) (7, 19, 24, 29).

DNA and protein sequence analysis. Sequence analysis was performed using the DNASTAR software package (Lasergene Inc., Madison, WI). The BLAST search tool was used for homology searches (1). Multiple alignments and phylogenetic trees were produced using the DNASTAR and MEGA3.1 software packages (15).

<table>
<thead>
<tr>
<th>ORF</th>
<th>Gene</th>
<th>Probable function or product</th>
<th>Homologous protein</th>
<th>Source</th>
<th>% Identity</th>
<th>Accession no.</th>
</tr>
</thead>
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<td>orf1</td>
<td>Truncated <em>cir</em>A</td>
<td>TonB-dependent receptor</td>
<td>CirA</td>
<td>Sphingomonas wittichii RW1</td>
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<tr>
<td>orf2</td>
<td>Truncated <em>top</em></td>
<td>Transposase</td>
<td>Transposase</td>
<td>Mesorhizobium lot MAFF303099</td>
<td>60</td>
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<td>Probable dehydrogenase</td>
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<td>40</td>
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<tr>
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<td>DbfA1</td>
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<tr>
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<td>Angular dioxygenase β subunit</td>
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<td>12</td>
<td>BAC75996</td>
</tr>
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</table>

**TABLE 1. Homology search analyses of the recovered ORFs from fluorene-degrading *Sphingomonas* sp. strain LB126**
The truncated gene product showed similarity to an extradiol dioxygenase with 42% protein identity to FlnE, a functional protein of Sphingomonas sp. strain LB126. The truncated gene was introduced into pET30f and expressed in E. coli BL21(DE3). Protein extracts from IPTG-induced cells were separated by SDS-PAGE. The cells overproduced two polypeptides with molecular masses of 45,000 and 14,000, which did not match exactly the predicted sizes of FlnA1 and FlnA2 as calculated from the deduced polypeptide sequences (49.5 and 19.4 kDa). Differences between the theoretical and apparent molecular masses upon SDS-PAGE gels were also observed for the DbfA1 and DbfA2 dioxygenase components from strain DBF63 (20). Significantly, it was found that the recombinant proteins were inactive and mostly insoluble (Fig. 4). When the recombinant strain was grown at 42°C up to an OD600 of 0.5 and subjected to IPTG induction at room temperature, a larger proportion of the FlnA1 and FlnA2 proteins was recovered in the soluble fraction (Fig. 4). In order to assess the enzymatic activity of FlnA1-FlnA2 in E. coli, biotransformation assays were carried out using induced cells incubated separately with fluorene, carbazole, dibenzofuran, dibenzothiophene, and dibenzox-p-dioxin, as well as with representative PAHs. Water-soluble oxidation products released into the culture medium were extracted and analyzed using GC-MS. The detection of fluorene oxidation products demonstrated that the recombinant enzyme was active in vivo (Table 2), suggesting that it recruited unspecific electron carriers from the host for function. When strain BL21(DE3)(pET30f), which lacked FlnA1-
FlnA2, was incubated with the same PAHs under identical conditions, no oxidation product could be detected, demonstrating that FlnA1-FlnA2 was indeed responsible for fluorene transformation (Table 2).

**Substrate range of FlnA1-FlnA2.** The substrate range of FlnA1-FlnA2 was investigated and compared with those of the well-studied angular dioxygenases dibenzofuran 4,4a-dioxygenase (DFDO) from *Terrabacter* sp. strain DBF63 (20) and carbazole 1,9a-dioxygenase (CARDO) from *Pseudomonas resi-novorans* CA10 (31, 37). When fluorene was used as a substrate, three oxidation products could be detected (Table 2). The major product was identified as 1-hydro-1,1a-dihydroxy-9-fluorenone (63%) based on the *m/z* fragment pattern of its mass spectrum, which was identical to that of the DFDO-mediated oxidation product of fluorene (20, 28) and the CARDO-mediated oxidation product of 9-hydroxyfluorene (43). Interestingly, CARDO does not yield the angular dioxygenation product 1-hydro-1,1a-dihydroxy-9-fluorenone when fluorene is used as a substrate (43). Fluorenol-dihydriodiol (7%) and dihydroxyfluorenone (29%) were also produced by FlnA1-FlnA2 from strain LB126. The latter product was not formed by DFDO. Fluorenol-dihydriodiol probably resulted from the spontaneous transformation of 1-hydro-1,1a-dihydroxy-9-fluorenone, since this product was not detected after shorter incubations. 9-Hydroxyfluorenone is likely oxidized into 9-fluorenone by a nonspecific dehydrogenase from *E. coli*. Indeed, we also observed such spontaneous oxidation upon the incubation of 9-hydroxyfluorenone with the control strain BL21(DE3)(pET30f) lacking the *flnA1-flnA2* insert. This result suggests that a dehydrogenase is not essential for the transformation of 9-hydroxyfluorenone into 9-fluorenone but that it may be required in vivo to catalyze the reaction at a reasonable rate. 1-Hydro-1,1a-dihydroxy-9-fluorenone also accumulated when 9-hydroxyfluorenone or 9-fluorenone was used as a substrate, showing that FlnA1-FlnA2 is involved in at least two steps in fluorene catabolism (Table 2). Given the low level of activity of FlnA1-FlnA2 and the necessity of monooxygenation before angular dioxygenation can occur, 9-hydroxyfluorenone and 9-fluorenone are probably instantly consumed and are therefore not present for detection.

Three heteroatomic analogs of fluorene, i.e., dibenzofuran, carbazole, and dibenzothiophene, were tested as substrates for angular oxygenation. Dibenzofuran was transformed into 2,2′,3′-trihydroxybiphenyl by FlnA1-FlnA2, as previously found for DFDO (20) and CARDO (31). The initial attack occurred at the 4 and 4a carbon atoms, as put forward by Fortnagel et al. in 1989 (8). The dioxygenation of dibenzofuran produces a highly unstable hemiacetal product that could not be observed. Incubation with dibenzothiophene produced traces of dibenzothiophene sulfoxide and dibenzothiophene sulfone. These metabolites were previously identified as metabolic intermediates of dibenzothiophene degradation by *Brevibacterium* sp. strain DO (45), DFDO (28), and CARDO (31). Since FlnA1-FlnA2 was able to perform angular dioxygenation of fluorene and dibenzofuran, hydroxylation of dibenzothiophene sulfone at the angular position was expected. The level of activity of FlnA1-FlnA2 toward dibenzothiophene may have been too low to detect an angular dioxygenation product by GC-MS, although dibenzothiophene is degraded cometabolically by strain LB126 (46). No angular oxidation product was detected after incubation with carbazole, even though carbazole is a structural analogue of fluorene. Mono- and dihydroxy carbazole were the only oxidation products detected by GC-MS. DFDO from *Terrabacter* sp. strain DBF63 was not able to perform angular dioxygenation of carbazole either. The detection of monohydroxy carbazole suggests that FlnA1-FlnA2 transforms carbazole into the corresponding dihydrodiol by lateral dioxygenation. Resnick et al. reported that carbazole dihydrodiols are unstable and spontaneously form monohydroxy carbazole by dehydration (35). CARDO released 2′-aminophenyl-2,3-diol upon the angular oxidation of carbazole (31). Previously, the crystal structure of CARDO bound to carbazole was resolved and a molecular mechanism of angular dioxygenation for carbazole was proposed (2). Given the low level of protein sequence identity between CARDO and FlnA1 (16%), FlnA1-FlnA2 may be missing the amino acid sequence and hence the corresponding structural specificity for the angular dioxygenation of carbazole. The incubation of FlnA1-FlnA2 with dibenzo-3,4-dioxin yielded 2,3,2′-trihydroxydiphenyl ether via angular dioxygenation based on the described *m/z* fragments from DFDO and CARDO.

Since *Sphingomonas* sp. strain LB126 is able to use phenanthrene, fluoranthene, and anthracene in cometabolic degradation with pyruvate (46), we tested whether FlnA1-FlnA2 would attack these PAHs. *cis*-9,10-Dihydroxy-9,10-dihydrophenanthrene, which was previously identified as a product formed by pyrene dioxygenase from *Mycobacterium* sp. strain 6PY1 (22), was detected as the major oxidation product of phenanthrene. Interestingly, *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene, which is produced in the catabolic pathway of known phenanthrene degraders, including siphingomonads (7, 34, 49), was not formed. Monohydroxyphenanthrene was detected in small amounts (4%) and may have resulted from the spontaneous dehydration of the corresponding dihydrodiol. In contrast, DFDO did not produce any metabolite when incubated in the presence of phenanthrene.
When FlnA1-FlnA2 was incubated with fluoranthene, trace amounts of monohydroxyfluoranthene could be detected. Anthracene yielded three metabolites. The major compound could be identified as cis-1,2-dihydroxy-1,2-dihydroanthracene by comparison to the oxidation product formed by Phn1 from *Sphingomonas* sp. strain CHY-1 (7). Trace amounts of monohydroxyanthracene were also present. CARDO produced the same metabolites, but DFDO did not. Moreover, a second putative anthracene-diol was detected. Its mass spectrum was similar to that of cis-1,2-dihydroxy-1,2-dihydroanthracene, but the retention time was different. Since no angular attack on anthracene is possible without preliminary monoxygenation, we suggest that this compound may be cis-2,3-dihydroxy-2,3-dihydroanthracene. This metabolite has not been produced by any other enzyme reported.
so far. When incubated with biphenyl or naphthalene, FlmA1-FlmA2 produced the expected metabolites, which were also reported previously for DFDO and CARDO (20, 31). Our results show that FlmA1-FlmA2 from strain LB126 is unique in that it shares characteristics with both DFDO and CARDO.

Quantitative analysis of water-soluble dihydrodiols formed by FlmA1-FlmA2. The catalytic activities of FlmA1-FlmA2 toward fluorene and other PAHs were compared by estimating the amount of di- or trihydroxylated products formed by strain BL21(DE3)(pET30f-FlmA1-FlmA2) after overnight incubation. Products were extracted and quantified as NBB derivatives by GC-MS analysis as described in Materials and Methods. Results showed that 1-hydro-1,1a-dihydroxy-9-fluorenone (retention time, 16.6 min) showed a relatively low level of activity toward naphthalene and other PAHs and heteroaromatics that are not oxidized by DFDO, the most closely related enzyme from Terrabacter sp. strain DBF63.

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