The fluorene catabolic linear plasmid in Terrabacter sp. strain DBF63 carries the β-ketoadipate pathway genes, pcaRHGBDCFIJ, also found in proteobacteria

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Terrabacter sp. strain DBF63 is capable of degrading fluorene (FN) to tricarboxylic acid cycle intermediates via phthalate and protocatechuate. Genes were identified for the protocatechuate branch of the β-ketoadipate pathway (pcaR, pcaHGBDCFIJ) by sequence analysis of a 70 kb DNA region of the FN-catabolic linear plasmid pDBF1. RT-PCR analysis of RNA from DBF63 cells grown with FN, dibenzofuran, and protocatechuate indicated that the pcaHGBDCFIJ operon was expressed during both FN and protocatechuate degradation in strain DBF63. The gene encoding β-ketoadipate enol-lactone hydrolase (pcaD) was not fused to the next gene, which encodes γ-carboxymuconolactone decarboxylase (pcaC), in strain DBF63, even though the presence of the pcaL gene (the fusion of pcaD and pcaC) within a pca gene cluster has been thought to be a Gram-positive trait. Quantitative RT-PCR analysis revealed that pcaD mRNA levels increased sharply in response to protocatechuate, and a biotransformation experiment with Escherichia coli carrying both catBC and pcaD indicated that PcaD exhibited β-ketoadipate enol-lactone hydrolase activity. The location of the pca gene cluster on the linear plasmid, and the insertion sequences around the pca gene cluster suggest that the ecologically important β-ketoadipate pathway genes, usually located chromosomally, may be spread widely among bacterial species via horizontal transfer or transposition events.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a ubiquitous class of hydrophobic organic compounds commonly found in the environment because of industrial activities, such as coal gasification, wood preservation, liquefaction and fuel refinement. Given that PAHs have toxic, mutagenic and carcinogenic properties, their persistence in the environment is of major concern. Therefore, the degradation of PAHs by diverse aerobic bacteria has been extensively studied in the last two decades (Cerniglia, 1992; Habe & Omori, 2003; Kanaly & Harayama, 2000; Pinyakong et al., 2003; Shuttleworth & Cerniglia, 1995). The microbial degradation of fluorene (FN), classified by the US Environmental Protection Agency as one of sixteen priority PAHs (Keith & Telliard, 1979), has been studied because FN is a major constituent of coal derivatives (e.g. about 7-6% of creosote PAHs; Grifoll et al., 1995a). Furthermore, FN and a number of its alkyl derivatives have been detected in vehicle
Strain DBF63 was originally isolated as a bacterium capable of utilizing both dibenzofuran (DF) and FN (Montejo et al., 1993). Initially, dbfA1 and dbfA2 were isolated from strain DBF63 as genes encoding novel terminal oxygenase components of the angular dioxygenase for DF degradation (Kasuga et al., 2001), and later, the genes were revealed to be located within an FN-catabolic gene cluster (Habe et al., 2004). The FN-catabolic genes, designated dbf-fln (involved with the degradation of FN to phthalate) and pht (involved with the degradation of phthalate to protocatechuate), were located on linear plasmids pDBF1 and pDBF2 (approx. 160 and 190 kb, respectively), and pDBF2 is thought to be derived from pDBF1 (Nourji et al., 2002a). The presence of protocatechuate catabolic genes in strain DBF63, which are required for the complete degradation of FN to TCA cycle intermediates, is supported by the fact that strain DBF63 can grow with protocatechuate as its sole carbon and energy source.

Two major catabolic pathways for protocatechuate have been proposed. The first is the meta-cleavage pathway initiated by protocatechuate 4,5-dioxygenase, and the second is the beta-ketoacid pathway initiated by protocatechuate 3,4-dioxygenase (Dagley et al., 1960; Fig. 1). The beta-ketoacid pathway, in particular, is considered to be a good model system for studying mechanisms of evolution in an ecologically important catabolic pathway (Buchan et al., 2004; Parke et al., 2000). Following initial ortho-cleavage by protocatechuate 3,4-dioxygenase (PcaHG), five additional enzymes, i.e. beta-carboxy-cis-cis-muconate cycloisomerase (PcaB), gamma-carboxymuconolactone decarboxylase (PcaC), beta-ketoacid enol-lactone hydrolase (PcaD), beta-ketoacid succinyl CoA transferase (PcaIJ) and beta-ketoacidyl CoA thiolase (PcaF), convert the ring cleavage product to TCA cycle intermediates (Fig. 1). In the case of the proteobacteria, this pathway is biochemically conserved among phylogenetically different strains, but operon organization, regulatory proteins, coinducers molecules and transport proteins for the pathway are remarkably diverse, most likely reflecting subtle aspects of niche adaptation (Buchan et al., 2004; Parke, 1997; Parke et al., 2000). In contrast, there is still little information available concerning protocatechuate catabolic gene clusters from actinobacteria (Eaton, 2001; Eulberg et al., 1998; Iwagami et al., 2000). The protocatechuate branch of the beta-ketoacid pathway (involving pca gene products) has only been functionally analysed in two systems, i.e. Rhodococcus sp. (Eulberg et al., 1998; Patraushan et al., 2005) and Streptomyces sp. (Iwagami et al., 2000). Analysis of the pca genes in Terrabacter may, therefore, provide a better perspective on the evolution of the beta-ketoacid pathway in actinobacteria, if this strain has such genes.

In this study, to elucidate all of the FN-degradative genes in Terrabacter sp. strain DBF63, we sequenced the 70 kb DNA region containing the already identified dbf-fln and pht genes. Interestingly, the pca genes, usually located chromosomally, were located on the linear plasmid. This is believed to be the first description of pca genes from actinobacteria, in which the beta-ketoacid enol-lactone-hydrolase and gamma-carboxymuconolactone decarboxylase enzymes are encoded by separate genes, pcaD and pcaC, respectively.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Terrabacter sp. strain DBF63 was routinely cultivated as previously reported (Kasuga et al., 1997). Briefly, this strain was cultivated in a carbon-free mineral medium (CFMM) supplemented with each substrate at a final concentration 0.1% (w/v) at 30 °C. The CFMM contained, per litre of distilled water: 2.2 g Na2HPO4, 0.8 g KH2PO4, 3.0 g NH4NO3, 0.2 g MgSO47H2O, 50 mg CaCl2, 2H2O, 50 mg FeCl3.6H2O. Strains of Escherichia coli were cultivated using LB broth, 2% YT medium or Terrific broth, as described by Sambrook et al. (1989). Ampicillin sodium salt, chloramphenicol and IPTG were added to the media at final concentrations of 100 μg ml−1, 30 μg ml−1 and 1 mM, respectively, when necessary.

**DNA manipulations.** Preparation of the total DNA of strain DBF63 and recombinant DNA manipulations were performed as reported previously (Kasuga et al., 1997). DNA fragments were purified using an EZNA gel extraction kit (Omega Bio-tek) according to the manufacturer’s instructions. Other DNA manipulations were carried out as described by Sambrook et al. (1989), and commercially available enzymes and kits were used as indicated by their manufacturers.

**Sequencing and annotation of the 70 kb DNA region of the linear plasmid.** Shotgun sequencing of (i) two previously isolated cosmid clones (designated pCC4 and pCC19) that contain the phtA1 gene (Kasuga et al., 2001), and (ii) one cosmold clone (designated pCC31) that contains the flanking region of the pCC4 insert (this study) was performed by Dragon Genomics. Fragmented, blunt-ended DNA was ligated into the SmaI site of pUC118. Determination of the nucleotide sequence was carried out by the chain-termination method. To identify ORFs, the nucleotide sequence was analysed with DNASIS-Mac software (version 3.7; Hitachi Software Engineering). Homology searches were carried out using the SWISS-PROT protein sequence database or the DDBJ, EMBL and GenBank nucleotide sequence databases with BLAST programs (Altschul et al., 1997). Results from the automated ORF prediction and functional assignment were manually controlled for the entire DNA contiguous sequence (70 kb). The nucleotide sequence was deposited at the DDBJ, EMBL and GenBank nucleotide sequence databases under the accession number AP008980.

**RT-PCR analysis.** Strain DBF63 was grown to mid-exponential phase in CFMM supplemented with FN, DF or protocatechuate solutions to a final substrate concentration of 0.1% (w/v). Both FN and DF were dissolved in dimethylformamide (DMF; 100 mg ml−1), and protocatechuate was dissolved in ethanol (100 mg ml−1). These solutions were sterilized by filtration with a 0.2 μm pore-size membrane filter, and 50 μl each solution was added to 5 ml CFMM. Two millilitres of each culture was then centrifuged, and total RNA was extracted from the harvested cells using a NucleoSpin RNA II purification kit (Macheray-Nagel), combined with RQ1 RNase-free DNAse (Promega), according to the manufacturer’s instructions. The gene-specific reverse primers used to synthesize cDNA were RT-Pr3R for...
PCAHG, RT-Pr5R for pcaDC, RT-Pr7Rb for pcaFJl and RT-Pr1R for pcaR (Table 2). cDNA of each gene was synthesized from 10 ng total RNA using SuperScript III reverse transcriptase (Invitrogen), according to the manufacturer’s instructions. The primers used are listed in Table 2. After the cDNA samples were properly diluted, PCR was performed as follows: (i) for PCAHG amplification, 95 °C for 10 min and 5 s, 60 °C for 10 s, and 72 °C for 30 s, for 40 cycles; (ii) for PCA DC amplification, 95 °C for 10 min and 30 s, 50 °C for 30 s, and 72 °C for 90 s, for 40 cycles; (iii) for PCAFJl amplification, 95 °C for 10 min and 5 s, 55 °C for 30 s, and 72 °C for 30 s, for 40 cycles; (iv) for pcaR amplification, 95 °C for 10 min and 30 s, 55 °C for 30 s, and 72 °C for 90 s, for 40 cycles.

Quantitative RT-PCR analysis. After a 4 day cultivation of strain DBF63 in 100 ml CFMM supplemented with DF, the cells were centrifuged and then washed twice with CFMM. The washed cells were resuspended in CFMM to an OD600 of approximately 0.6, and 5 ml portions of the resulting cell suspension were transferred to four test
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic</th>
<th>Reference or source</th>
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<td><strong>Strains</strong></td>
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<tr>
<td>Terrabacter sp. DBF63</td>
<td>FN utilization (FN⁺)</td>
<td>Monna et al. (1993)</td>
</tr>
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<td>Terrabacter sp. DBF63W</td>
<td>FN⁻ mutant of DBF63</td>
<td>Kasuga et al. (1997)</td>
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<td>E. coli JM109</td>
<td>recA1, endA1, gyrA96, thi-1, hisD17, supF4, relA1, Δ(lac-proAB)/F[trdA36, proAB+, lacI², lacZAM15]</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
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<td><strong>Cosmids</strong></td>
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<td>pCC12</td>
<td>Ap⁺, Km⁺, SuperCos1 (Stratagene) with BamHI insert of DBF63 DNA</td>
<td>Kasuga et al. (2001)</td>
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<td><strong>Plasmids</strong></td>
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<td>pUC18/19</td>
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<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pUC118/119</td>
<td>Ap⁺, lacZ; pMB9 replicon</td>
<td>Yanisch-Perron et al. (1985)</td>
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<td>pSTV28/29</td>
<td>Cm⁺, lacZ; pMB9 replicon</td>
<td>Takara Shuzo</td>
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<tr>
<td>pT7Blue(R)</td>
<td>Ap⁺, T-vector</td>
<td>Novagen</td>
</tr>
<tr>
<td>pBluescript II KS(+)/-</td>
<td>Ap⁺, lacZ; pMB9 replicon</td>
<td>Stratagene</td>
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<td>pUCA811</td>
<td>Ap⁺, 3.2 kb EcoRI fragment from Pseudomonas resinovorans strain CA10 DNA inserted into EcoRI-digested pUC19; carries catBC</td>
<td>Nojiri et al. (2002b)</td>
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<td>pUCA832</td>
<td>Ap⁺, 1.9 kb Xhol–EcoRI fragment from pUCA811 inserted into Xhol/EcoRI-digested pBluescript II KS(+)/-; carries catBC, lac orientation</td>
<td>This study</td>
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<td>pDFS603</td>
<td>Ap⁺, 0.4 kb PCR product (using pCC12 as a template) inserted into HindIII/EcoRI-digested PSTV29; carries pcaC, lac orientation</td>
<td>This study</td>
</tr>
<tr>
<td>pDFS604</td>
<td>Ap⁺, 0.8 kb PCR product (using pCC12 as a template) inserted into HindIII/EcoRI-digested PSTV29; carries pcaD, lac orientation</td>
<td>This study</td>
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</table>

Construction of plasmids for pcaD, pcaC and catBC expression. The 820 bp and 415 bp DNA fragments containing pcaD and pcaC, respectively, were prepared by PCR using pCC12 (Kasuga et al., 2001) as the template. The primer sets used are listed in Table 2. PCR was carried out using LA Taq with GC buffer (Takara Shuzo) and PCR Thermal Cycler Dice (Takara Shuzo). The cycling conditions were as follows: 96°C for 1 min, followed by 30 cycles of 96°C for 30 s, 55°C for 30 s, and 72°C for 2 min, followed by 72°C for 6 min. The PCR products were cloned using the pT7Blue (R) vector (Novagen), and the nucleotide sequences of the PCR products were confirmed by sequencing. The clones were digested with both HindIII and EcoRI (sites derived from the primer, Table 2), and then the fragments were cloned between the HindIII and EcoRI sites of pSTV29 to give pDFS604 (carrying pcaD) and pDFS603 (carrying pcaC), respectively. To construct the expression plasmid for catBC genes, the 1.9 kb Xhol–EcoRI fragment of pUCA811 (Nojiri et al., 2002b) was cloned between the corresponding sites of pBluescript II KS(−) to give pUCA832 (carrying catBC). Resting cell reactions and analysis of the products. Transformation of E. coli cells with the constructed plasmids was performed as described by Hanahan (1983). An appropriate E. coli transformant was grown in 100 ml LB broth supplemented with ampicillin, chloramphenicol and IPTG, as appropriate, in a 500 ml flat-bottom flask at 30°C with reciprocal shaking at 120 strokes min⁻¹. The culture broth was centrifuged (4000 g), and the pellets were washed twice with 100 ml buffer (containing 2-2 g Na₂HPO₄, 0-8 g KH₂PO₄, and 3-0 g NH₄NO₃ per litre of distilled water; pH 7.0), and resuspended in the same buffer to an OD₆₆₀ of approximately 10. We added cis,cis-muconate (0-1 % w/v) to 5 ml resting cell suspension in test tubes and incubated the tubes at 30°C for 2 h. The reaction mixtures were acidified with 1 M HCl and extracted with 5 ml ethyl acetate. The extracts were analysed by GC-MS after derivatization with N-methyl-N-trimethylsilyl trifluoroacetamide. GC-MS analyses were performed with a JMS-Automass 150 GC-MS system (JEOL) fitted with a fused-silica chemically bonded capillary
RESULTS

Sequencing of the 70 kb DNA region of linear plasmid pDBF1

The shotgun sequencing of three cosmid clones produced a 70,631 bp contiguous sequence that included the 20,574 bp region previously analysed (Habe et al., 2003, 2004). In addition to the already determined ORFs (ORF1–ORF20), 57 additional ORFs (ORF21–ORF77) and one incomplete ORF (ORF78) were annotated (Fig. 2; see the Supplementary Table with the online journal). The mean G+C content of the 70,631 bp region was 68.6 %, close to that of the genome of strain DBF63 (70.5 %; Kasuga et al., 1997). (i) Protocatechuate catabolic genes. Based on a homology search (see the Supplementary Table), nine genes, i.e. pcaH (ORF56), pcaG (ORF55), pcaB (ORF54), pcaD (ORF53), pcaC (ORF52), pcaF (ORF51), pcaI (ORF50) and pcaJ (ORF49), were identified that apparently coded for the protocatechuate 3,4-dioxygenase β subunit (PcaH), protocatechuate 3,4-dioxygenase α subunit (PcaG), β-carboxy-cis,cis-muconate cyclosomerase (PcaB), γ-carboxymuconolactone decarboxylase (PcaC), β-ketoacidopate enol-lactone hydrolase (PcaD), β-ketoacidopate succinyl CoA transferase α subunit (PcaI), β-ketoacidopate succinyl CoA transferase β subunit (PcaI) and β-ketoacidopate CoA thiolase (PcaF), respectively (Fig. 1). The start codons (ATG or GTG) of all pca genes overlapped with the stop codon (TGA) of the preceding pca gene by 1 or 4 bp (data not shown), indicating that these nine genes were transcribed together. Residues known to be important for catalytic function were well conserved in the DBF63 Pca protein sequences, e.g. the residues involved in coordinating the nonheme Fe³⁺ (Tyr408, Tyr447, His460 and His462), as well as active-site residues (e.g. Thr12, Pro15, Tyr324, Trp400, Trp449, Arg457 and Ile491) in PcaHG of Acinetobacter sp. strain ADP1 (Orville et al., 1997; Vetting et al., 2000), and the cysteine residue demonstrated to be critical for hydrolysis activity (Cys123) in PcaD from Pseudomonas sp. strain B13 (Pathak et al., 1991). ORF57

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Name of primer</th>
<th>Nucleotide sequence*</th>
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<td>For RT-PCR</td>
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<td>pcaR</td>
<td>RT-Pr1R†</td>
<td>5’-CCGCTTGAAGTCGGAAGTAC-3’</td>
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<td>RT-Pr1F</td>
<td>5’-GCTGTCGCCCTTGAAC-3’</td>
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<td>RT-Pr3R†</td>
<td>5’-AGCTGAGAGCAGACTC-3’</td>
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<td></td>
<td>RT-Pr3F</td>
<td>5’-CAGCCCATGATGCAGYTC-3’</td>
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<td>pcaDC</td>
<td>RT-Pr5R†</td>
<td>5’-GAAGGGGATGACGCTAC-3’</td>
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<td>qRT-pcaDFb2</td>
<td>5’-TCGTCGCCCATCTGTC-3’</td>
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<td>pcaFIJ</td>
<td>RT-Pr7R†</td>
<td>5’-GAAGGGGATGACGCTAC-3’</td>
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<td>RT-Pr7Fb</td>
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<td>Intergenic region between pcaR and pcaH</td>
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<td>5’-ACGACCTCGAGGCATAG-3’</td>
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<td>pcaD</td>
<td>qRT-pcaDFb2†</td>
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<td>16S rDNA</td>
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<td>pcaD-F</td>
<td>5’-TTAAGCCTTAAAGGGCTGCAGCTAGGCTAGGCAGACCTG-3’</td>
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<td>pcaC</td>
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<td>pcaC-F</td>
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<td></td>
<td>pcaC-R</td>
<td>5’-AAGATTCTCATGTTTCCCTGCCGGCTGAAGGCTGGCGG-3’</td>
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</table>

*The italic, underlined and double-underlined sequences are an efficient Shine–Dalgarno sequence (Shine & Dalgarno, 1975), a HindIII site and an EcoRI site, respectively.
†The gene-specific reverse primers were used for synthesizing cDNA.
is transcribed divergently from pcaH, and encodes a protein with the helix–turn–helix motif at its N-terminal region and shares identities with the putative transcriptional regulators belonging to the IclR family, so we designated it pcaR.

(ii) Insertion sequences (ISs). We found several ORFs homologous to transposase genes or IS-like elements around the pca and dbf-fln gene clusters (Fig. 2). The deduced amino acid sequence of ORF58 located downstream of pcaR exhibited 100% similarity to that of the transposase-encoding gene in ISTesp2, found just upstream of the phl genes in strain DBF63 (Habe et al., 2003). We also determined that the incomplete ORF78 and its flanking region were homologous to ISTesp2 (Fig. 2).

The DNA sequence revealed that ORF68 and its flanking region (nucleotides 61 177–62 584; Fig. 2) were identical to ORF70 and its flanking region (nucleotides 64 306–65 713), and that the full-length of these repetitive elements was 1408 bp. The deduced amino acid sequences of ORF42, ORF68 and ORF70 showed 82, 84 and 84% overall lengthwise similarity, respectively, to the transposase of IS1601-A from Mycobacterium avium (see the Supplementary Table) and that the full-length of these repetitive elements was 1408 bp. The deduced amino acid sequences of ORF42, ORF68 and ORF70 showed 82, 84 and 84% overall lengthwise similarity, respectively, to the transposase of IS1601-A from Mycobacterium avium (see the Supplementary Table) and that the full-length of these repetitive elements was 1408 bp. The deduced amino acid sequences of ORF42, ORF68 and ORF70 showed 82, 84 and 84% overall lengthwise similarity, respectively, to the transposase of IS1601-A from Mycobacterium avium (see the Supplementary Table) and that the full-length of these repetitive elements was 1408 bp.

The polypeptide encoded by ORF67 and ORF66 showed 66–68% and 52–69% overall lengthwise similarity to the transposase subunits encoded by MC34 and MC35, respectively, from the plasmid pSD10 of Micrococcus sp. strain 28 (AY034092.1), and by PDB2.181 and PDB2.182, respectively, from plasmid pBD2 of Rhodococcus erythropolis strain.
BD2 (Stecker et al., 2003). This IS is potentially a member of the IS3 family. The 24 bp imperfect IR was found around the two ORFs, but a direct repeat, duplicated target site, was not observed in the immediate vicinity of the IR.

Transcriptional analysis of pca genes by RT-PCR

We used RT-PCR analysis to investigate whether pca genes were transcribed in strain DBF63 during its growth with FN. Total RNA was prepared from DBF63 cells grown with FN, DF or protocatechuate. As the pcaHGBDFIJ gene cluster has been shown, by RT-PCR, to be transcribed as an operon (data not shown), we only present here the results with the three primers specific for amplifying the pcaHG, pcaDC and pcaFIJ genes within this cluster (Table 2; Supplementary Fig. 1a with the online journal). Clear amplification of the appropriate portion of the respective pca genes (1.2 kb for pcaHG, 0.85 kb for pcaDC and 1.4 kb for pcaFIJ) was detected when RNA prepared from both FN- and protocatechuate-grown cells were used, whereas PCR products were not detected when RNA from DF-grown cells was used. No amplified products were detected in the negative control lacking reverse transcriptase (data not shown). These results indicate that pcaHGBDFIJ genes were transcribed in DBF63 cells grown with FN, as well as protocatechuate, suggesting that the genes are probably required for FN degradation in this strain. As strain DBF63 degrades DF via gentisate (Monna et al., 1993), it is reasonable that PCR products are not detected from cells grown on DF. In contrast, the pcaR gene was not specifically expressed during the degradation of FN or protocatechuate, and the same levels of PCR products were detected from RNA prepared from DBF63 cells grown on all three substrates (see Supplementary Fig. 1a). This result suggests that the pcaR gene may be constitutively expressed in this strain. RT-PCR with primers specific for amplifying the intergenic region between pcaR and pcaH (Table 2) produced no PCR products of the expected size (310 bp; data not shown).

Quantitative RT-PCR

To analyse the expression pattern of pca genes, the pcaD mRNA levels in protocatechuate-induced or non-induced DBF63 cells, within 1 h of induction, were determined by quantitative RT-PCR with SYBR Green. Transcriptional levels were normalized with 16S rRNA as an internal control. As shown in Supplementary Fig. 1(b) (left panel), the pcaD mRNA in cells with protocatechuate was about 21-fold more abundant than in cells with ethanol (control). Similarly, the pcaD mRNA levels in FN-induced or non-induced DBF63 cells were examined (see Supplementary Fig. 1b, right panel), and expression levels 1.7-fold higher were observed in cells with FN. The differences in water solubility of these inducer substrates, and the solvent types, may have had some effect on induction levels. In addition, incubation times longer than 1 h would produce higher induction levels in FN-induced cells. Nevertheless, the patterns of sharp and gradual increase in the pcaD mRNA levels in response to protocatechuate and FN, respectively, indicate that protocatechuate itself or its metabolite, e.g. β-ketoadipate, is the inducer of Pca enzymes.

β-Ketoadipate enol-lactone hydrolase (PcaD) activity

We constructed the plasmids pUCA832 (carrying catBC) and pDFS604 (carrying pcaD; Table 1), and performed a biotransformation experiment with cis,cis-muconate using E. coli cells harbouring both pUCA832 and pDFS604. E. coli JM109 cells carrying both pUCA832 and pSTV29 were used as a control. GC-MS analysis of products from cis,cis-muconate after treatment with N-methyl-N-trimethylsilyl-trifluoroacetamide, gave a mass spectrum that exhibited fragment ions at m/z 378 (M+, 6), 361 (27), 317 (6), 286 (16), 259 (6), 231 (21), 169 (59), 147 (39), 125 (8) and 73 (100), where the numbers in parantheses represent relative intensity, detected with a retention time of 8.4 min. This fragmentation pattern of the trimethylsilyl derivative of the metabolite was identical to that of authentic β-keto-diacid (data not shown). In contrast, β-ketoadipate was not detected in the control sample (data not shown). Instead, the trimethylsilyl derivative of β-ketoadipate enol-lactone was tentatively identified because the mass spectrum (m/z) 286 (M+, 14), 271 (5), 169 (100), 147 (19) and 73 (100) and the retention time (6.3 min) were consistent with those of the product when we performed biotransformation experiments with protocatechuate using E. coli cells harbouring both pAROS23 (carrying pcaHGB; Parke, 1995) and pDFS603 (carrying pcaG; see Fig. 1). The above result indicates that the pcaD gene encodes β-ketoadipate enol-lactone hydrolase.

DISCUSSION

In this study, we examined the gene organization of a 70 631 bp DNA region derived from the FN-catabolic linear plasmid pDBF1 of Terrabacter sp. DBF63 and found it to include β-ketoadipate pathway genes (Fig. 2). The β-ketoadipate pathway is widely distributed among diverse soil microbes, and plays an important role in the metabolism of naturally occurring aromatic compounds and environmental pollutants. The pathway genes are usually located chromosomally in proteobacteria (Harwood & Parales, 1996), although there are some exceptions such as pWW174 from Acinetobacter calcoaceticus (Winstanley et al., 1987) and pSymB from Sinorhizobium meliloti (Finan et al., 2001). Similarly, in actinobacteria the pca genes are not located on plasmids. For example, Iwagami et al. (2000) reported that the pca genes from Streptomyces coelicolor strain A3(2) are located on the genetically stable region of its chromosome. In addition, the pca genes are not located on the linear megaplasmid pICP of Rhodococcus opacus strain 1CP (König et al., 2004) nor on linear plasmids (e.g. pRHL1) of Rhodococcus sp. strain RHA1 (Patrauchan et al., 2005). Hence, the underlying importance of the pathway for the metabolism of aromatic compounds that are produced abundantly by plants may make the pathway genes typically...
chromosomally encoded. In the present study, however, we discovered that strain DBF63 possessed the pca genes on the linear plasmid pDBF1. As we did not obtain any PCR products for pca genes (pcaH, pcaG, pcaD or pcaC) when total DNA from strain DBF63W, which was obtained by the continuous culture of the wild-type strain on nutrient broth and lacks the FN-degrading linear plasmid, was used as a template (data not shown), this pca gene cluster was not located (duplicated) on the chromosome. Moreover, strain DBF63W did not grow with protocatechuate as a carbon source (data not shown), suggesting that the protocatechuate catabolic gene cluster is only located on the plasmid. Recently, other DF-degrading bacteria, *Terrabacter* sp. strain DFA1 and *Janibacter* sp. strain DFA10, both of which possess *dbfA1A2* gene homologues, were isolated, and the gene organizations of the regions flanking the *dbfA1A2* gene homologues were analysed. Sequence comparisons of these DNA regions among strains DBF63, DFA1 and DFA10 showed that the organizations of both *pht* and *dbf-fln* gene clusters were completely conserved (Noumura et al., 2004). These results strongly suggest that pDBF1 of strain DBF63 is a transmissible plasmid, and may play an important role in the distribution of FN-degradation genes. Together with the fact that many IS-like elements and transposase genes are flanked with the pca gene cluster, it suggests that the β-ketoadiapate pathway genes may be acquired via transposition events and subsequently distributed among actinobacteria via the horizontal transfer of plasmids.

Eulberg et al. (1998) first found a pcaL gene that encoded a merged enzyme with β-ketoadiapate enol-lactone hydrolase and γ-carboxymuconolactone decarboxylase in the rhodococcal pca gene cluster, although these two enzymes are encoded by separate genes, pcaD and pcaC, respectively, in most proteobacteria (Buchan et al., 2004). In addition, Iwagami et al. (2000) identified the pcaL gene homologue in the streptomycete pca gene cluster (strain 2065). Recent genome sequence analyses also show that *S. coelicolor* strain A3(2) (SCO939128), *Streptomyces avermitilis* MA-4680 (AP005027.1) and *Corynebacterium glutamicum* strain ATCC13032 (BX927155) possess the pcaL gene. This fusion of the two proteins into one is predicted to be beneficial to the stabilization of the enzyme itself, and to the efficient delivery of substrates and products by minimizing the distance between the active sites of each protein (Eulberg et al., 1998). Furthermore, it is hypothesized that the separate pcaD and pcaC gene arrangement of proteobacteria may be more ancient, and that the presence of a fused pcaL gene is a Gram-positive trait (Eulberg et al., 1998; Iwagami et al., 2000). However, in this study we found that pDBF1 carried the separate pcaD and pcaC genes that had not been found in the pca gene cluster of actinobacteria (Fig. 3). The pcaD gene was expressed in both FN- and protocatechuate-induced DBF63 cells (Supplementary Fig. 1), and the PcaD enzyme exhibited its activity as a single protein, indicating that PcaD functions during FN or protocatechuate degradation by strain DBF63. Therefore, the presence of the pcaL gene is not quite a Gram-positive trait. Considering that the

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**Fig. 3.** Gene organization of pca gene clusters from actinobacteria [*Terrabacter* sp. strain DBF63 (accession number AP008980), *S. coelicolor* strain A3(2) (AL079355) and *Rhodococcus opacus* strain 1CP (AF003947)]. As a comparison, a pca gene organization from proteobacteria [*Acinetobacter* sp. ADP1 (L05770)] is shown under the dotted line. Putative regulatory genes (pcaR, pcaU and SCO6704) are indicated by grey shading. Gene designations are given in Supplementary Table 1. pcaK is a proposed aromatic-acid-transport gene. The organization of pca gene clusters from *S. coelicolor* strain A3(2) and *Streptomyces* sp. strain 2065 were identical (Iwagami et al., 2000). Also, the pca gene organizations of *Rhodococcus opacus* strain 1CP and *Rhodococcus* sp. strain RHA1 were identical (Patrauchan et al., 2005).
merged pcaL gene also exists in Ralstonia metallidurans (a β-proteobacteria) and Caulobacter crescentus (an α-proteobacteria) within the pca gene cluster (Jimenez et al., 2002), whether the Pca enzyme arrangement of actinobacteria is more evolved than that of proteobacteria may be irrelevant.

The structure of the pca gene cluster from strain DBF63 was compared with that from S. coelicolor strain A3(2) and Rhodococcus opacus strain 1CP (Fig. 3). The gene organizations from pcaH to pcaL. (pcaD and pcaC in strain DBF63) were highly similar among these strains. However, the locations of pcaF, pcaIJ and pcaR genes were markedly different. In strain DBF63, pcaFJ was located at the end of the cluster, whereas in strain A3(2), pcaIJF was located upstream of pcaH. In these two species, both structural gene clusters pcaHGBDCFII and pcaIJFHGBL seemed to be located in a single operon, and the transcriptional regulatory gene, pcaR, was present upstream of the respective operons (Fig. 3). In contrast, pcaIJ of strain 1CP was transcribed divergently from other pca genes, and interestingly, pcaR seemed to be cotranscribed with the preceding pcaHGBL genes as a single operon because pcaR overlapped pcaL by 4 bp (Eulberg et al., 1998; Fig. 3). These differences in gene arrangement affect the regulation of pca genes. In strain DBF63, the expression of the pcaR gene is probably not elicited by protocatechuate (Supplementary Fig. 1a), and is likely to be constitutive. However, the expression of the pcaR gene in strain 1CP, which may be cotranscribed with the structural genes, is likely to be induced. We have not yet established a procedure for gene knockouts in Terrabacter sp. strain DBF63, but if this can be achieved, the detailed transcriptional mode of not only pca genes, but also both dbf-fln and pht genes in concert with the putative corresponding regulators (PcaR, FlnR and PhtR, respectively), can be performed. It would be interesting to compare the regulatory systems among pca gene clusters in strains DBF63, A3(2) and 1CP.

In conclusion, this report is believed to be the first to (i) elucidate all catalytic pathway genes for the conversion of FN to TCA cycle intermediates, (ii) find the genes encoding the protocatechuate branch of the β-ketoacipitate pathway on a linear plasmid, and (iii) find separate pcaD and pcaC genes, but not the merged pcaL gene, within the pca gene cluster of an actinobacteria species. The study of further examples of actinobacterial pca gene clusters, and comparisons of both the organization and regulatory systems of these pca operons may illuminate an evolutionary trait in actinobacteria that could be a consequence of the distinctive selection pressures faced by organisms maintaining the β-ketoacipitate pathway.

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