Evolution of the IncP-7 carbazole-degradative plasmid pCAR1 improves survival of its host *Pseudomonas fluorescens* Pf0-1 in artificial water microcosms

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In our previous study, *Pseudomonas fluorescens* Pf0-1L, harbouring the IncP-7 carbazole-degradative plasmid pCAR1::rfp, was shown to be undetectable within 5 days post-inoculation in carbazole-contaminated artificial freshwater microcosms containing several plasmid-free bacteria in addition to Pf0-1L(pCAR1::rfp). Fourteen days after the inoculation, carbazole degraders become detectable. Here, we revealed that these isolates were not pCAR1 transconjugants, but Pf0-1L(pCAR1::rfp) mutants, based on RFLP and BOX-A1R-based repetitive extragenic palindromic-PCR (BOX-PCR) analysis. Notably, the mutants displayed more rapid initiation of carbazole degradation than the parent strain Pf0-1L(pCAR1::rfp). The mutants were unable to degrade anthranilate due to a 163 bp deletion in the antA gene, which was overcome by their transformation with a wild-type antABC-expressing plasmid. Quantitative RT-PCR analysis indicated that the transcriptional induction of carbazole-, anthranilate- and catechol-degradative genes was comparable in both parent and mutant strains. The deletion mutants became dominant in the artificial water microcosm. The mutation caused anthranilate to accumulate instead of catechol, a toxic compound for the parent strain, and may be beneficial to host survival in artificial microcosms.

INTRODUCTION

Degradative plasmids enable their hosts to degrade various xenobiotic compounds (e.g. toluene, xylene, naphthalene), and are transmitted between different bacteria in the natural environment by conjugative transfer (Nojiri et al., 2004, 2009; Ogawa et al., 2004; Williams et al., 2004; Shintani et al., 2010a). Plasmid pCAR1 is a conjugative catabolic plasmid belonging to the incompatibility (Inc)P-7 group (Shintani et al., 2005a, b, 2006). The original host of pCAR1 is *Pseudomonas resinovorans* CA10 (Ouchiyama et al., 1993; Nojiri et al., 2001), and the entire sequence of pCAR1 has been determined (Maeda et al., 2003). Although there are pCAR1 derivatives, identified as pCAR1.1 and pCAR1.2 (previously pCAR2 from another carbazole degrader; Takahashi et al., 2009b), due to a single nucleotide substitution (synonymous substitution) or the presence of another copy of ISPre1 (Takahashi et al., 2009b), we recognize them as being the same plasmid and describe them as pCAR1 in this paper.

pCAR1 confers upon host cells the ability to degrade carbazole, a nitrogen-containing heterocyclic aromatic compound. Carbazole is converted to catechol via the upper pathway enzymes CarABC (conversion of carbazole to anthranilate) and AntABC (conversion of anthranilate to catechol) (Fig. 1a). CarABC and AntABC are encoded by the *car* and *ant* operons on pCAR1 (Fig. 1a). Catechol is then metabolized by CatABC and lower-pathway enzymes encoded on the host chromosome in most bacteria of the genus *Pseudomonas* (Harwood & Parales, 1996; Fig. 1a). Because catechol is host-dependently metabolized, the carbazole-metabolic activity may have become differentiated in different host strains. Indeed, the growth rate of *Pseudomonas fluorescens* Pf0-1(pCAR1) in mineral medium with carbazole as the sole carbon source is markedly lower.
Fig. 1. (a) The carbazole-catabolic pathway in most *Pseudomonas* strains harbouring pCAR1 and the benzoate-catabolic pathway of *Pseudomonas* strains. Solid and dashed arrows indicate conversions catalysed by Car and Ant enzymes, encoded by pCAR1, or Ben/Cat enzymes, encoded on the host chromosome. CarAaAcAd, carbazole 1,9a-dioxygenase; CarBaBb, 2′-aminobiphenyl-2,3-diol 1,2-dioxygenase; CarC, 2-hydroxy-6-oxo-6-(2′-aminobiphenyl)-hexa-2,4-dienoate hydrolase; CarD, 2-hydroxyocta-2,4-dienoate hydratase; CarE, 4-hydroxy-2-oxovalerate aldolase; CarF, acetaldehyde dehydrogenase (acylating); AntABC, anthranilate 1,2-dioxygenase; TCA cycle, tricarboxylic acid cycle; BenABC, benzoate 1,2-dioxygenase; BenD, cis,cis-dihydroxycyclohexa-3,5-diene-1-carboxylate dehydrogenase; CatA, catechol 1,2-dioxygenase; CatB, cis,cis-muconate lactonizing enzyme; CatC, muconolactone isomerase. (b) Organization of the pCAR1 car and ant genes and the ben-cat gene clusters on the *P. fluorescens* Pf0-1 chromosome. The cat gene cluster of *P. putida* KT2440 is also shown. Black, grey and white pentagons indicate regulatory genes, catabolic genes and other unrelated ORFs, respectively, and rectangles indicate insertion sequences. Arrows from anthranilate, benzoate and cis,cis-muconate in (a) indicate that they are effectors of the transcriptional regulators AntR, CatR and BenR, respectively. Parallel vertical bars (antA, antR, carAa upstream, catB1 and catB2) indicate regions amplified by qRT-PCR.
than those of other host strains, including Pseudomonas putida KT2440(pCAR1) and Pseudomonas aeruginosa PAO1(pCAR1) (Takahashi et al., 2009a). This difference stems from differential regulation of the transcription of the *catBCA* genes that encode catechol-degrading enzymes in Pfo-1 and other hosts (Takahashi et al., 2009a). In brief, *cat* genes in Pfo-1 are not induced by a metabolic intermediate of the catechol-degradative pathway but by benzoate (Fig. 1b; Takahashi et al., 2009a), whereas those in other hosts are induced by cis,cis-muconate, a metabolic intermediate in the catechol-degradative pathway (Fig. 1b; Cowles et al., 2000). The imbalance in metabolic capacities between the upper and lower carbazole-degradation pathways in Pfo-1(pCAR1) cells grown on carbazole results in the excessive accumulation of catechol, which becomes darker by autoxidation and polymerization, and is toxic to the host cells (Takahashi et al., 2009a). Interestingly, the appearance of mutants with *pCAR1*-derivative plasmids that have deleted carbazole-degradative operons(s) (pCAR1d, which lacks *antR–antABC* and *pCAR1Δ2*, which lacks *antR–antABC* and *car*) prevents the accumulation of catechol and relieves the above metabolic imbalance (Takahashi et al., 2009a).

Previously, we monitored how pCAR1 hosts that include such an ‘inappropriate’ carbazole degrader behave in artificial microcosms (Shintani et al., 2008b, 2010b). We constructed three different types of artificial microcosms, consisting of sterilized buffer (MRA), filtered river water (MRB2) and filtered pond water (MRC2), with 12 strains of *Pseudomonas* and three strains of non-*Pseudomonas* bacteria (Shintani et al., 2008b, 2010b). All were supplemented with carbazole and inoculated with pCAR1 hosts, and then changes in the residual carbazole, host survival and conjugative transfer of the plasmid were monitored (Shintani et al., 2008b, 2010b). Pfo-1L(pCAR1::rfp), a derivative of strain Pfo-1(pCAR1), was constructed to detect plasmid transfer more easily. This strain carries the *lacI* gene on its chromosome, and its plasmid was tagged with a reporter gene encoding red fluorescent protein (RFP), which is expressed downstream from a LacI-repressible promoter (Shintani et al., 2008a). As expected, Pfo-1L(pCAR1::rfp) disappeared immediately after inoculation in the MRA microcosm, probably because of its low growth rate on carbazole (Fig. 2; Shintani et al., 2010b). In contrast, although Pfo-1L(pCAR1::rfp) disappeared within 5 days of inoculation, carbazole degradators became detectable after approximately 14 days in the other artificial water microcosms (MRB2 and MRC2) (Fig. 2; Shintani et al., 2010b). Notably, carbazole degradation was detected to coincide with the recovery of the degraders (Shintani et al., 2010b). In the present study, we analysed these strains genetically and compared their carbazole-degradative abilities to examine the mechanism(s) behind their emergence in the microcosms.

**METHODS**

**Bacterial strains, media and culture conditions.** The bacterial strains used in this study are listed in Table 1. Note that *P. fluorescens* Pfo-1L(pCAR1::rfp) has RFP (RFP-expressing gene) on pCAR1 and *lacI* on the Pfo-1 chromosome to monitor conjugal transfer of the plasmid (Shintani et al., 2008a, 2010b). *Escherichia coli* DH5α was used as a host strain for the preparation of plasmids pUC19 and pBBR1MCS-5, and their derivatives. Bacteria were grown in L-medium (10 g tryptone peptone l⁻¹, 5 g yeast extract l⁻¹, and 10 g NaCl l⁻¹) (Sambrook & Russell, 2001) or nitrogen plus mineral medium-4 (NMM-4) (Shintani et al., 2005a), with carbazole, anthranilate or benzoate as the sole source of carbon and energy. Each 10 % (w/v) stock solution of the compounds (carbazole was dissolved in DMSO, the others were dissolved in distilled water) was added to NMM-4 to obtain a final concentration of each carbon source of 0.1 % (w/v). L-medium and NMM-4 with carbazole or anthranilate were solidified with 1.6 % (w/v⁻¹) agar, generating L-broth (LB), CAR or ANT plates, respectively. Colonies on CAR plates with clearing zones around the colony were considered to be viable carbazole degraders. Growth profiles of carbazole degraders were determined as follows. The degraders were grown in 5 ml L-medium for 15 h and then washed with NMM-4. To obtain an initial turbidity at 600 nm of 0.05, 100 ml NMM-4 containing 0.1 % (w/v) carbazole was inoculated with the washed cells. The cultures were grown at 30 °C on a rotary shaker at 200 r.p.m., and cell growth was monitored by counting c.f.u. ml⁻¹ on LB. Bacterial conjugation was performed as described previously (Shintani et al., 2005b).

**Standard DNA manipulations.** Total DNA was extracted from each bacterial strain as described by Ausubel et al. (1990). Extraction of DNA from the bacterial community in each artificial microcosm was performed with a NucleoSpin Tissue kit (Machery-Nagel) according to the manufacturer’s instructions. PCR analysis was performed using TaKaRa Ex Taq Polymerase (Takara Bio) with appropriate primers (Sigma Genosys). pCAR1(68675)-F (5'-TTGGGATTTACCGGACGTCGAGTGAAG-3') and pCAR1(69570)-R (5'-TCGGATCCCTATCAACGATT3') were used for the amplification of the flanking regions of pCAR1 repA, the 68 675–69 570 region on pCAR1, whose complete sequence had previously been determined (accession no. AB088420; Maeda et al., 2003). For the amplification of the *lacI* gene on the Pfo-1L(pCAR1::rfp) chromosome, primers lacI-F (5'-TGGGATTTACCGGACGTCGAGTGAAG-3') and lacI-R (5'-GATTAATGAATCGGCCAAC-3') were used. Primers antI7111-F (5'-GCTGCGCCTTGTAGCAGCAGC-3') and antABC-R (5'-TCAAGCTCCTGCCCATCCCTCCGGGT3') were used to amplify the regions surrounding the 163 bp deletion in the *antA* gene. PCR was performed using the following conditions: 98 °C for 2 min, followed by 30 cycles of 98 °C for 10 s, 60 °C for 20 s and 72 °C for 30 s. Primers 27F and 1378R (Weisburg et al., 1991; Heuer et al., 1997) were used for amplification of bacterial 16S rRNA genes. The conditions used were 96 °C for 2 min, followed by 30 cycles of 96 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min. RFLP analysis was performed by digesting the 16S rRNA gene with *MnlI* (New England BioLabs) and *HaeIII* (Takara Bio) for 3 h at 37 °C, and then separating the resulting fragments on 2 % agarose gels. BOX-ARF-based repetitive extragenic palindromic-PCR (BOX-PCR) was performed with primer BOXA1R (5'-GTATGGATTTACCGGACGTCGAGTGAAG-3') and BOXA1R (5'-GCATTAATGAATCGGCCAAC-3') as primers. Amplification was performed using the following conditions: 95 °C for 2 min, followed by 16 min incubation at 65 °C. Nucleotide sequencing was performed by FASMAC (Kanagawa, Japan) or by using an ABI Prism 310 genetic analyser (Applied Biosystems) according to the manufacturer’s instructions. The partial sequences of pUCantABC_PF, pUCantABC_5EPB3 and pUCantABC_5EP124 (Table 1) were determined. Southern hybridization analysis was performed as described previously (Shintani et al., 2005b) using *antA* and ORF19 probes, prepared by PCR-amplifying the 6193–6547 and 27 444–27 851 regions of pCAR1 (accession no. AB088420).
Quantitative RT-PCR (qRT-PCR). Precultures of Pf0-1L(pCAR1::rfp), 5EP83, 5EP124 and 5EP126 were grown in 5 ml L-medium for 15 h, and then washed with NMM-4. To obtain an initial turbidity at 600 nm of 0.05, 100 ml NMM-4 containing 0.1 % (w/v) of each carbon source was inoculated with the washed cells. The cultures were grown at 30°C on a rotary shaker at 120 r.p.m. for 6 h. Total RNA was extracted from the resulting culture using RNAprotect Bacteria Reagent (Qiagen) and a NucleoSpin RNA II kit (Macherey-Nagel). DNA was degraded using RQ1 RNase-free DNase (Promega), and total RNA was purified using a NucleoSpin RNA II kit. qRT-PCR was performed according to the method of Takahashi et al. (2009a). Briefly, 20 μl reverse-transcription reaction mixture was prepared using the following: 1 μg total RNA, 75 ng random primers (Invitrogen), 200 U SuperScript II (Invitrogen), 40 U RNaseOUT (Invitrogen), 1 x First Strand Buffer (Invitrogen), 10 mM DTT and 0.5 mM deoxynucleoside triphosphates (Toyobo). The univ16S-F/-R primer set was used to measure transcription of the rrn genes (reference data for qRT-PCR) of genus *Pseudomonas* bacteria, and antA-F/-R, antR-F/-R and ORF9-F/-R primer sets were used to measure the transcription of *antA*, the transcriptional regulator gene *antR*, and the upstream region of *carA*, respectively (primer sequences given in Miyakoshi et al., 2009). Similarly, the catB1-F/-R and catB2-F/-R primer sets with pCAR1 were used to measure the *catB1* and *catB2* genes on the Pf0-1 chromosome (primer sequences given in Takahashi et al., 2009a). Each primer set was designed to amplify about 100 bp of product. The cDNA quantification was performed using an ABI 7300 Real-Time PCR system and Power SYBR Green PCR Master Mix (Applied Biosystems). After quantification of cDNA by real-time PCR, a melting-curve analysis was performed to verify the amplification specificity.

**antABC complementation assay.** A *Pst*I (4192 nt on pCAR1, accession no. AB088420)–*Hin*III (8061 nt on pCAR1, accession no. AB088420) fragment containing the wild-type *antABC* genes was subcloned into pBBR1MCS-5 from pSCos708 (Nojiri et al., 2001). The resulting plasmid, pBBRantABC, was introduced into Pf0-1L(pCAR1::rfp), 5EP83, 5EP124 and 5EP126 through conjugation with *E. coli* S17-1(λpir). pBBR1MCS-5 was similarly introduced into each strain as a control. After their isolation, transconjugants were streaked out on ANT plates and their growth was assessed.

**Detection of anthranilate in cultures of mutants by GC-MS.** Overnight cultures of Pf0-1L(pCAR1::rfp), 5EP83, 5EP124 and 5EP126 in L-medium were harvested to obtain a turbidity at 600 nm of 5.0. Each of the harvested cells was resuspended in 5 ml NMM-4 medium supplemented with carbazole, whose final concentration was 0.1 % (w/v). Part (500 μl) of each culture was sampled after 18, 45 and 120 h and centrifuged (15 000 g, 2 min, room temperature). Anthranilate in the resulting supernatants was methylated as follows. Five microlitres of each supernatant was mixed with 95 μl 10 % (w/w) trifluoro(methanol)boron (Sigma) and incubated at 90°C for 20 min. Then, 1.9 ml distilled water was added, and methyl esters were extracted with 5 ml ethyl acetate. The ethyl acetate extract was evaporated and the residue was redissolved in 100 μl methanol. The standards were prepared by saturating 1 ml methanol with anthranilic acid in the same manner. Following addition of 10 μl 1 % (w/v) trifluoro(methanol)boron in methanol, the samples were heated at 90°C for 20 min. The supernatants were extracted as above, and methyl esters were measured by GC-MS. The samples with added anthranilic acid served as standards. The retention times and the mass of the major fragment were matched with those of the standards by GC-MS.

**Detection of anthranilate in cultures of Pf0-1L(pCAR1::rfp).** The anthranilate produced in each culture was measured. The anthranilate concentration was calculated based on the peak area of the standards. The anthranilate concentration was expressed as μg/μl of supernatant. The anthranilate concentration at each time point was compared with the concentration of the control sample (SUP-1L). The difference in anthranilate concentration between the experimental and control samples was calculated and expressed as a percentage of the control value.
18 h sample of the parent strain Pf0-1L(pCAR1 : :
Anthranilate (1, 2, 5, 10, 25, 50, 100 and 250 p.p.m.) was used as an
Microbiology and three from MRC2), we performed PCR analysis using
post-inoculation (Fig. 2; at least three strains from MRB2
Identification of carbazole degraders that
reappeared in artificial water microcosms
30 or 42 days
RESULTS
Identification of carbazole degraders that
reappeared in artificial water microcosms
After isolation of the carbazole degraders 29 or 42 days
post-inoculation (Fig. 2; at least three strains from MRB2 and three from MRC2), we performed PCR analysis using
Primers for the amplification of a specific pCAR1 region
(repA-flanking region). RFLP analyses and BOX-PCR
patterns of the isolates were identical to those of the
parent strain P. fluorescens Pf0-1L(pCAR1 : : rfp) (data not
shown). We also confirmed that these strains possessed the
lacI gene (data not shown), which had previously been
introduced on the Pf0-1L(pCAR1 : : rfp) chromosome
(Shintani et al., 2010b). Therefore, these isolates were
not pCAR1 transconjugants, but derivatives of Pf0-
rfp
P. fluorescens
P. fluorescens
P. fluorescens
P. fluorescens
P. fluorescens
P. fluorescens
P. putida
P. putida
Plasmids or cosmids
pBBR1MCS-5
pBBRandABC
pCAR1 : : rfp
pCAR1 : : rfp : : 5EP83
pUCos708
pUC19
pUCantABC : : 5EP124
pUCantABC : : 5EP126
pUCantABC : : 5EP83
pUCantBC : : PF
pUCFLG1
pUCFLG2
Anthranilate was extracted in 2 ml ethyl acetate. The resulting extracts
were analysed by GC-MS using a JMS-K9 system (JEOL) equipped
with an Agilent 7683 Series Automatic Liquid Injector (Agilent
Technologies) and an InertCap capillary column (0.25 mm x 15 m,
0.25 µm, GL Science). Column temperature was programmed to
increase at a rate of 16 °C min⁻¹ from 80 °C (initial temperature) to
160 °C, and then at 60 °C min⁻¹ to 280 °C (final temperature),
with holds of 2 and 3 min at the initial and final temperatures, respectively.
Anthranilate (1, 2, 5, 10, 25, 50, 100 and 250 p.p.m.) was used as an
external standard (correlation coefficient >0.99; data not shown).
Relative anthranilate levels were determined from the ratio of the
peak area for mel 119 of the extract from each mutant to that of the
18 h sample of the parent strain PFO-1L(pCAR1 : : rfp).

Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
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<tr>
<td>E. coli DH5α</td>
<td>F⁻, 808d, lacZΔM15, Δ(lacZYA-argF), U169, endA1, recA1, hsdR17(rK mC), deoR, thi-1, supE44, λ, gyrA96, relA1</td>
<td>Toyobo</td>
</tr>
<tr>
<td>E. coli S17-1 (λpir)</td>
<td>Tn², Sm², recA, thi, pro, hsdR⁻ M⁺, RP4 : 2-Tc : Mu: Km Tn7 λpir</td>
<td>Simon et al. (1983); Compeau et al. (1988); Silby et al. (2009)</td>
</tr>
<tr>
<td>P. fluorescens Pf0-1</td>
<td>Sequenced strain</td>
<td>Shintani et al. (2010b)</td>
</tr>
<tr>
<td>P. fluorescens Pf0-1L(pCAR1 : : rfp)</td>
<td>PFO-1 with lacI, pCAR1 : : rfp</td>
<td>This study</td>
</tr>
<tr>
<td>P. fluorescens 5EP83</td>
<td>P. fluorescens Pf0-1L(pCAR1 : : rfp) derivative strain isolated from artificial water microcosm</td>
<td>This study</td>
</tr>
<tr>
<td>P. fluorescens 5EP124</td>
<td>P. fluorescens Pf0-1L(pCAR1 : : rfp) derivative strain isolated from artificial water microcosm</td>
<td>This study</td>
</tr>
<tr>
<td>P. fluorescens 5EP126</td>
<td>P. fluorescens Pf0-1L(pCAR1 : : rfp) derivative strain isolated from artificial water microcosm</td>
<td>This study</td>
</tr>
<tr>
<td>P. putida KT2440</td>
<td>Sequenced strain</td>
<td>Bagdasarian et al. (1981); Nelson et al. (2002)</td>
</tr>
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<td>P. putida KT2440RG</td>
<td>Derivative strain of KT2440, spontaneous Rif⁻ with introduced Gm' gene</td>
<td>Shintani et al. (2003a)</td>
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<tr>
<td>pBBR1MCS-5</td>
<td>Gm', lacZα mob; compatible with IncP, IncQ and IncW plasmids</td>
<td>Kovach et al. (1995)</td>
</tr>
<tr>
<td>pBBRandABC</td>
<td>pBBR1MCS-5 containing 8 kb Pst–HindIII from pSCos708</td>
<td>This study</td>
</tr>
<tr>
<td>pCAR1 : : rfp</td>
<td>pCAR1 containing rfp gene on 197,493 nt of pCAR1</td>
<td>Shintani et al. (2008a)</td>
</tr>
<tr>
<td>pCAR1 : : rfp : : 5EP83</td>
<td>pCAR1 : : rfp derivative plasmid of 5EP83</td>
<td>This study</td>
</tr>
<tr>
<td>pUCos708</td>
<td>Ap', Km', SuperCosI(Stratagene) with EcoRI insert of CA10 DNA</td>
<td>Nojiri et al. (2001)</td>
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<td>pUC19 containing 8 kb Pst–HindIII fragment on pCAR1 : : rfp : : 5EP126</td>
<td>This study</td>
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<td>pUC19 containing 8 kb Pst–HindIII fragment on pCAR1 : : rfp : : 5EP83</td>
<td>This study</td>
</tr>
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<td>pUCantBC : : PF</td>
<td>pUC19 containing 5.6 kb EcoRI–SphI fragment (fragment I in Fig. 5b) on pCAR1 : : rfp : : 5EP83 containing antiA and partial region of ORF14</td>
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<td>pUCFLG1</td>
<td>pUC19 containing 3.7 kb EcoRI–SphI fragment (fragment I in Fig. 5b) on pCAR1 : : rfp : : 5EP83 containing partial regions of ORF24 and ORF9</td>
<td>This study</td>
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Catabolic phenotypes of the parent strain and isolates from microcosms
Carbazole-degradative activity was assessed by whether the liquid culture medium turned clear, because carbazole is not completely dissolved in the medium. The carbazole

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degradation by the isolates from MRC2 (5EP83, 5EP124, 5EP126) was clearly detected at around 30 h, while the detection of degradation by Pf0-1L(pCAR1::rfp) took more than 146 h (data not shown). Degradation by the three isolates from MRB2 was no faster than that by Pf0-1L(pCAR1::rfp) (data not shown), and therefore we focused on the analysis of the isolates from MRC2. Fig. 3 shows the growth curves of strains Pf0-1L(pCAR1::rfp), 5EP83, 5EP124 and 5EP126. Although the lag phases to initiate carbazole degradation of the three isolates were much shorter than that of Pf0-1L(pCAR1::rfp), the final c.f.u. counts of the three strains were lower than that of Pf0-1L(pCAR1::rfp) (Fig. 3). The culture of Pf0-1L(pCAR1::rfp) turned brown, while the cultures of the isolates from MRC2 did not (data not shown). To elucidate the reasons for these phenomena, we subjected the three MRC2 strains (hereafter referred to as mutants) to further investigation.

Catabolic phenotypes for other carbon sources were also compared between the parent strains and the three mutants from MRC2. Notably, none of the mutants was able to grow on medium supplemented with anthranilate as a sole carbon source (data not shown), while the parent strain Pf0-1L(pCAR1::rfp) was. It was noteworthy that dark-brown pigments were generated in the medium with the parent strain (data not shown). This was because Pf0-1 is not able to metabolize catechol effectively in the absence of benzoate, an inducer of the ben-cat genes (Fig. 1b), so that the catechol may have accumulated following the conversion of anthranilate into catechol. Given that both the parent strain and the mutants were, in contrast, able to use benzoate as a sole source of carbon (data not shown), chromosomal gene products that degrade benzoate and catechol are apparently active in them (see Fig. 1b).

Genetic and transcriptional analyses of genes encoding enzymes for carbazole degradation in the mutants

To assess whether the loss of the ability to degrade anthranilate in the mutants was caused by mutation(s) in pCAR1 or host chromosomes, plasmids from the parent strain (pCAR1::rfp) and the mutants 5EP83, 5EP124 and 5EP126 (named as pCAR1::rfp_5EP83, pCAR1::rfp_5EP124 and pCAR1::rfp_5EP126, respectively) were introduced into P. putida KT2440RG, which is unable to use anthranilate as a sole carbon source. While transconjugants carrying pCAR1::rfp grew on ANT plates, those carrying plasmids from mutants did not (data not shown). This finding suggests that the genes in pCAR1::rfp that control anthranilate degradation were mutated in the mutants (i.e. AntABC was defective). We also compared the genetic structures of the ben-cat gene flanking regions (Fig. 1) of the mutants with those of the parent strain. Southern hybridization analysis revealed no change around these DNA regions (data not shown). To assess whether the mutants had change(s) in regulatory systems for expression of carbazole-degradative genes, qRT-PCR analyses were performed on the genes encoding carbazole-degrading enzymes (car, ant and cat genes). It was found that the car and ant genes were induced by carbazole or anthranilate, in both the parent strain and the mutants (Supplementary Fig. S1). antR encodes a transcriptional regulator of the car and ant operons (Urata et al., 2004). Although transcriptional levels of antR in 5EP124 and 5EP126 under several conditions were lower than those in other strains (Supplementary Fig. S1), the transcription of antA and catAa was induced to a similar extent (Supplementary Fig. S1). Therefore, the differences in antR transcript levels may not affect the metabolism of carbazole or its intermediate compounds. The cat genes (catB1 and catB2) were induced by benzoate, but not by carbazole or anthranilate (Supplementary Fig. S1). In conclusion, we were unable to detect drastic differences in the induction of the pCAR1 car and ant genes and the chromosomal cat genes between the mutants and the parent strain. In complementation assays performed using the antABC genes, three mutants transfected with pBBRantABC turned the ANT plates dark brown, while those with a control vector (pBBR1MCS-5) did not (Supplementary Fig. S2). It should be noted that colonies of mutants with pBBRantABC could not be detected on the ANT plates, because Pf0-1 is not able to metabolize catechol effectively, as mentioned above. The change in plate colour to dark brown is caused by autoxidation and polymerization of the accumulated catechol.
catechol, while the lack of colony formation may have resulted from damage caused by excessive accumulation of catechol (Supplementary Fig. S2). These findings indicate that the failure of the mutants to degrade anthranilate was caused solely by the mutation(s) in the pCAR1 :: rfp antABC genes.

To identify mutated regions of the antABC genes in the mutants, a HindIII–PstI fragment containing the antABC genes and the regions flanking them was cloned into a pUC19 vector (yielding pUCantABC_5EP83, pUCantABC_5EP124 and pUCantABC_5EP126), as was the corresponding region of Pf0-1L(pCAR1 :: rfp) (yielding pUCantABC_PF). Nucleotide sequence analysis showed that an identical 163 bp region of the antA gene (7279–7441 nt, accession no. AB088420) was missing in every mutant (Fig. 4a). The regions in pCAR1 upstream of antA and carAa are highly conserved (Fig. 4a), possibly due to the one-ended transposition of ISPre1 (Nojiri et al., 2001). Note that the missing 163 bp sequence was not present in the carAa upstream region in every strain (i.e. in the mutants, both upstream regions now lacked the 163 bp sequence; Fig. 4a). Indeed, PCR analysis using primers designed to amplify the regions surrounding the deletion [antA(7111)-F and antABC-R] revealed only a single band in the mutants, and two bands in the parent strain (Fig. 4b). To assess whether the mutants could convert anthranilate to catechol, we compared the levels of anthranilate in carbazole cultures of mutant and parent strains. As expected, about five- to 50-fold more anthranilate accumulated in the mutant cultures than in the parent strain culture (Supplementary Fig. S3).

### Inversion between tnpA1 and tnpA2 in pCAR1 :: rfp_5EP83

Analysis of the antA flanking regions of pCAR1 :: rfp and plasmids from mutants by Southern hybridization revealed a different size in the case of pCAR1 :: rfp_5EP83 (Fig. 5). By using antA and ORF9 probes to detect tnpA2- and tnpA1-containing DNA fragments, the hybridization signals from EcoRI-, SphI- and double-digested pCAR1 :: rfp_5EP83 were of sizes different from those of pCAR1 :: rfp (Fig. 5a). Differences were only detected in pCAR1 :: rfp_5EP83 and not in the other plasmids (data not shown). Two signal bands (about 9 and 10 kb) were detected on SphI-digested 5EP83 total DNA with an ORF9 probe, but only a single band was detected on EcoRI-digested or EcoRI/SphI double-digested 5EP83 total DNA (Fig. 5a). Given the nucleotide sequences of tnpA1 and tnpA2, and the fact that their flanking regions (antA upstream region and partial region of ORF9; Fig. 4a) are identical to each other, DNA inversion could have occurred in pCAR1 :: rfp_5EP83. We cloned a 5.6 kb EcoRI–SphI fragment (fragment I) containing the antA

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**Fig. 4.** (a) Organization of the flanking regions of antA and ORF9 in pCAR1 :: rfp and its derivatives pCAR1 :: rfp_5EP83, pCAR1 :: rfp_5EP124 and pCAR1 :: rfp_5EP126. Grey and white pentagons indicate genes encoding degradative enzymes and other ORFs, respectively, and rectangles indicate ISPre1 insertion sequences. Identical DNA sequences containing ISPre1, the promoters (P ant) and parts of ORF9/antA are highlighted in grey, and were probably generated by one-ended transposition of ISPre1 from the antA upstream region (Nojiri et al., 2001; Miyakoshi et al., 2006). A 163 bp section of the antA gene was missing in pCAR1 :: rfp_5EP83, pCAR1 :: rfp_5EP124 and pCAR1 :: rfp_5EP126, although this region was not present in ORF9 of every plasmid. The deletion resulting in disruption of antA due to a frame-shift mutation and the disrupted antA (∆antA) are shown by a pentagon with a dotted outline. Black triangles indicate the primer sets used to amplify the regions flanking the deleted sequence. (b) Gel electrophoresis analysis of the PCR amplicons generated using the primers shown in (a) and total DNA from inoculated and recovered strains.
upstream region and a 3.5 kb EcoRI–SpfI fragment (fragment II) containing a partial region of ORF9 on pCAR1::rfp_5EP83 into pUC19 (yielding pUCFLG1 and pUCFLG2, respectively), and then determined the nucleotide sequences of the terminal regions of their inserts. As expected, fragment I contained antA and a partial region of ORF14, while fragment II contained partial regions of ORF9 and ORF24 (Fig. 5b). Importantly, the suggested DNA inversion would reasonably explain the different Southern hybridization signals detected with pCAR1::rfp_5EP83, except for the above-mentioned 10 kb signal band on SpfI-digested total DNA of 5EP83 with the ORF9 probe (Fig. 5a, b). These results indicate that a DNA inversion occurred between tnpA1 and tnpA2 in pCAR1::rfp_5EP83, although the relationship between this DNA inversion and antA deletion was unclear. Unfortunately, we are currently not able to explain why we detected the 10 kb signal band.

To determine whether other regions of pCAR1::rfp_5EP83 were deleted, DNA–DNA hybridization was performed using pCAR1 tiling array chips (Miyakoshi et al., 2009), which are able to detect deletions of >10 bp. Using this technique, we detected a deletion in pCAR1::rfp_5EP83. The deleted sequence was located to the inner region of the antA gene (7281–7432 nt, accession no. AB088420) and coincided with the 163 bp deletion (7279–7441 nt, accession no. AB088420) detected by sequencing (see Supplementary Results, Supplementary Table S1).
Deletion mutants became dominant in the artificial microcosms

To determine exactly when the 163 bp deletion in antA gene had occurred, total DNA previously extracted from the artificial water microcosms at each sampling point (Shintani et al., 2010b) was subjected to PCR analyses using the primer set antA(7111)-F and antABC-R to detect the deletion. While two bands were consistently detected in the MRA microcosms during the monitoring period (Fig. 2a), the intensity of the signal from the larger band (corresponding to the intact antA gene) decreased in parallel with the appearance of carbazole degraders in the MRB2 and MRC2 microcosms (Fig. 2a). These results indicate that antA deletion mutants became dominant in the MRC2 microcosms from 42 days post-inoculation. The recovery of carbazole degraders as a result of the 163 bp deletion in the antA gene occurred coincidentally with the initiation of carbazole degradation in MRC2 microcosms (Fig. 2). Unfortunately, we were unable to isolate mutants with different growth rates on carbazole from the MRB2 samples. However, considering that the smaller band (corresponding to the deleted antA gene) increased similarly in the MRB2 samples (Fig. 2a), mutants carrying the 163 bp antA deletion probably also emerged in the MRB2 microcosms, coincident with carbazole degradation.

**DISCUSSION**

P0-1 is an ‘inappropriate’ host for pCAR1 from the viewpoint of carbazole degradation because of its failure to express genes encoding catechol-degrading enzymes in the presence of carbazole. We previously found pCAR1 to be structurally unstable in P0-1(pCAR1) in pure culture with carbazole as the sole carbon source (Takahashi et al., 2009a). In the late stage of this pure culture, host P0-1 strains harbouring pCAR1-derivative plasmids became dominant that had lost carbazole-degradation genes (car, antABC, antR), or both antABC and antR, most likely due to homologous recombination between two identical ISpre1 insertion sequences (Takahashi et al., 2009a). In this study, mutants with a 163 bp deletion in the antA inner region arose and were selected in artificial microcosms (MRC2), and they initiated carbazole degradation more rapidly than the parent strains. Although we were unable to isolate mutants from MRB2 microcosms, the deletion mutants came to dominate both artificial microcosms, MRB2 and MRC2 (Fig. 2a). It is possible that a large population of the parent strain still remained in the microcosms, and that by accident, no mutants were isolated from MRB2.

Taking account of the carbazole-degrading enzymes encoded by pCAR1 (Fig. 1), it seems likely that the mutants were able to use carbazole as the sole carbon source without a functional antA gene because they carry, on pCAR1, the carDEF genes, which encode enzymes involved in the conversion of 2-hydroxypenta-2,4-dienoate to acetyl-CoA (Fig. 1a; Nojiri et al., 2001). The reason why the final c.f.u. values of the mutants in their pure cultures were lower than that of the parent strain (Fig. 3) was probably because only half of the carbon atoms of carbazole could have been utilized in the mutants (Fig. 1, meta-cleavage pathway via CatDEF; Nojiri et al., 2001). It should be noted that catechol is known rapidly to form a brown pigment by a non-enzymic autoxidation reaction that is dependent on catechol concentration, and this pigment is toxic even for catechol degraders (Park et al., 2004). In contrast, anthranilate, which accumulated in the mutants (Supplementary Fig. S3), is an important intermediate of tryptophan metabolism that may be less toxic than catechol. Indeed, anthranilate is a major accumulated compound in carbazole degraders (Nojiri & Omori, 2007). Avoiding the accumulation of catechol may have enhanced the survival of the deletion mutants in the artificial microcosms with respect to the parent strain P0-1L(pCAR1::rfp).

Considering that antA-ISpre1 (tnpA2) (7424–8871 nt, accession no. AB088420) and the reverse complement of ISpre1 (tnpA1-ORF9) (25288–26717 nt, accession no. AB088420) exhibit 100% sequence identity (in a 1430 bp region; Fig. 4a), the inversion mutations we detected may have occurred through homologous recombination of the region containing the two copies of ISpre1. As for the 163 bp deletion in the antA gene, it was difficult to explain how it might have occurred; however, one possibility is a homologous recombination between the inner regions of antA and ORF9. pCAR1 carries three (or four) copies of ISpre1, single copies of ISpre2, ISpre4 and IS1162, and two copies of ISpre3 (Maeda et al., 2003; Takahashi et al., 2009b). In previous studies, we isolated derivatives of pCAR1 from its original host P. resinovorans CA10 (pCAR1A1, pCAR1A3) (Uratu et al., 2004) and P0-1 (pCAR1A, pCAR1d, pCAR1SUC1, pCAR1SUC2, pCAR1SUC3) (Takahashi et al., 2009a). These plasmids carry deletions as a result of homologous recombination between two copies of an insertion sequence. Although these derivatives of pCAR1 were not detected in MRA, MRB2 or MRC2 by PCR analysis (data not shown), these insertion sequences may have an important role in the evolution of pCAR1.

A comparison of the plasmid structures of the mutants from the artificial microcosms with those of pCAR1d (lacks antR-antABC) and pCAR1A2 (lacks antR-antABC and car), previously found in P0-1(pCAR1) pure cultures, showed that genes necessary for anthranilate degradation were commonly lost (Fig. 6). This suggests that avoiding excessive catechol (or brown pigment) accumulation may improve the survival of the host P0-1. While antR was missing from pCAR1d and pCAR1A2, it was retained by pCAR1 in the mutants from the microcosms (Fig. 6). AntR is an AraC/XylS-family regulator that induces the car and ant genes in the presence of its own inducer, anthranilate (Fig. 1b; Urata et al., 2004; Miyakoshi et al., 2006). The absence of antR slows the degradation of carbazole. Because of the absence of strains capable of efficiently metabolizing catechol, it may be important to delay carbazole degradation in pure cultures of P0-1(pCAR1) to avoid excessive catechol (or brown pigment) accumulation. In contrast, systems
for inducing the car operon in response to anthranilate are retained in the mutants from the microcosms (Supplementary Fig. S1). It should be noted that there were many other anthranilate- (or catechol-) metabolizing bacteria in the microcosms, including P. resinovorans CA10dm4 and P. aeruginosa PAO1 (Shintani et al., 2008b, 2010b). Although these strains were also able to degrade catechol, excess catechol was toxic even for them if the final concentration was more than 0.1% (w/v) (data not shown). Thus, degradation rates for carbazole and anthranilate, as well as their concentrations, may be the key factors for catechol accumulation or degradation. These strains are also able to metabolize anthranilate efficiently and may have prevented catechol from accumulating excessively and from forming brown pigment. Indeed, while pure liquid cultures of Pf0-1(pCAR1) containing carbazole turned darker (Takahashi et al., 2009a), carbazole-containing MRB2 and MRC2 microcosms did not. There may have been no requirement to slow the rate of carbazole degradation in the artificial water microcosms because of the presence of other anthranilate- or catechol-metabolizing bacteria. On the other hand, if only mutants with a DNA deletion in the antA gene were present and no other strains, including anthranilate or catechol degraders, it would not necessarily be beneficial from the viewpoint of carbon utilization because they would make use of only part of the carbazole as a carbon source. It is therefore possible that the deletion in the antA gene may not occur in the pure culture of Pf0-1(pCAR1), but only in the artificial microcosms. The direction of evolution of pCAR1 in the host Pf0-1 may have been affected by the presence of other anthranilate-metabolizing bacteria due to differences in catechol metabolism in the culture. To examine the mechanisms of bacterial (plasmid) genome evolution, it will be necessary to compare the fitness of Pf0-1 strains harbouring various pCAR1 derivatives in the presence and absence of other strains. The results of the present study highlight the importance of comparing the behaviour of plasmids and their hosts when grown on different carbon sources and in different environments, and in the presence of other bacteria.

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