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.

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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 (Ouchiya *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

Abbreviations: BOX-PCR, BOX-A1R-based repetitive extragenic palindromic-PCR; qRT-PCR, quantitative RT-PCR; RFP, red fluorescent protein.

The GenBank/EMBL/DDBJ accession numbers for the partial sequences of pUCantABC_PF, pUCantABC_5EP83 and pUCantABC_5EP124 of *P. fluorescens* are AB571236–AB571239.

Four supplementary figures, a supplementary table and Supplementary Results are available with the online version of this paper.

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-hydroxypenta-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*-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 Pf0-1 and other hosts (Takahashi *et al.*, 2009a). In brief, *cat* genes in Pf0-1 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 Pf0-1(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 operon(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). Pf0-1L(pCAR1::rfp), a derivative of strain Pf0-1(pCAR1), was constructed to detect plasmid transfer more easily. This strain carries the *lacI* gene on its chromosome, and its pCAR1 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, Pf0-1L(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 Pf0-1L(pCAR1::rfp) disappeared within 5 days of inoculation, carbazole degraders 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*

Pf0-1L(pCAR1::rfp) has *rfp* (RFP-expressing gene) on pCAR1 and *lacI* on the Pf0-1 chromosome to monitor conjugative transfer of the plasmid (Shintani *et al.*, 2008a, 2010b). *Escherichia coli* DH5α (Toyobo) was used as a host strain for the preparation of plasmids pUC19 and pBRR1MCS-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 (Macherey-Nagel) according to the manufacturer's instructions. PCR analysis was performed using TaKaRa ExTaq Polymerase (Takara Bio) with appropriate primers (Sigma Genosys). pCAR1(68675)-F (5'-TTGGGATTACGGGACT-GCT-3') and pCAR1(69570)-R (5'-TCGGATGCCTATCAACGATT-3') 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 Pf0-1L(pCAR1::rfp) chromosome, primers *lacI*-F (5'-TGTTGGTGTGTC-GATGGTAGAA-3') and *lacI*-R (5'-GCATTAATGAATCGGCCAAC-3') were used. Primers *antA*(7111)-F (5'-CGTCGCTTTTGTAGCAC-CAG-3') and *antABC*-R (5'-TCAGACCATCCCTAGCCTGT-3') 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 *MnII* (New England BioLabs) and *HaeIII* (Takara Bio) for 3 h at 37 °C, and then separating the resulting fragments on 2% agarose gels. BOX-A1R-based repetitive extragenic palindromic-PCR (BOX-PCR) was performed with primer BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3'; Versalovic *et al.*, 1994) using the following conditions: 95 °C for 7 min, and then 30 cycles of 94 °C for 1 min, 51 °C for 1 min and 65 °C for 8 min, followed by a 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_5EP83 and pUCantABC_5EP124 (Table 1) were determined. Southern hybridization analysis was performed as described previously (Shintani *et al.*, 2005b) using *antA* and ORF9 probes, prepared by PCR-amplifying the 6193–6547 and 27 444–27 851 regions of pCAR1 (accession no. AB088420).

Fig. 2. Behaviour of pCAR1, its host strain and other strains in artificial water microcosms inoculated with *P. fluorescens* Pf0-1L(pCAR1::rfp). Please note that (b) and (c) show the reference data from Shintani *et al.* (2010b). Samples were taken 0, 5, 13, 29 and 42 days post-inoculation. (a) PCR analysis using primer sets designed to amplify the *antA* flanking region shown in Fig. 4(a). Numbers indicate sampling day [as well as sampling time points in (b) and (c)]. M, DNA marker. The larger band corresponds to the intact *antA* gene, while the smaller band is the *carAa* upstream region and the *antA* gene with a 163 bp deletion. (b) Changes in carbazole (CAR) levels. Grey circles indicate the amount of carbazole remaining in the absence of bacterial inoculation, and black circles the amount of carbazole following bacterial inoculation. (c) Changes in the numbers of carbazole degraders. Data represent the mean $\pm$ SD of three independent experiments.

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 RNeasy Protect 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 μ l reverse-transcription reaction mixture was prepared using the following: 1 μ g total RNA, 75 ng random primers (Invitrogen), 200 U SuperScript II (Invitrogen), 40 U RNaseOUT (Invitrogen), 1 $\times$ 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 *carAa*, 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)–*Hind*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(λ pir). 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 μ l) 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 μ l 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

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>E. coli</i> DH5 α	F ⁻ , ϕ 80d, <i>lacZ</i> Δ M15, Δ (<i>lacZYA-argF</i>), <i>U169</i> , <i>endA1</i> , <i>recA1</i> , <i>hsdR17</i> (r _K ⁻ m _K ⁺), <i>deoR</i> , <i>thi-1</i> , <i>supE44</i> , λ , <i>gyrA96</i> , <i>relA1</i>	Toyobo
<i>E. coli</i> S17-1 (λ <i>pir</i>)	Tm ^r , Sm ^r , <i>recA</i> , <i>thi</i> , <i>pro</i> , <i>hsdR</i> ⁻ M ⁺ , RP4:2-Tc:Mu: Km Tn7 λ <i>pir</i>	Simon <i>et al.</i> (1983)
<i>P. fluorescens</i> Pf0-1	Sequenced strain	Compeau <i>et al.</i> (1988); Silby <i>et al.</i> (2009)
<i>P. fluorescens</i> Pf0-1L(pCAR1::rfp)	Pf0-1 with <i>lacI</i> ^r , pCAR1::rfp	Shintani <i>et al.</i> (2010b)
<i>P. fluorescens</i> 5EP83	<i>P. fluorescens</i> Pf0-1L(pCAR1::rfp) derivative strain isolated from artificial water microcosm	This study
<i>P. fluorescens</i> 5EP124	<i>P. fluorescens</i> Pf0-1L(pCAR1::rfp) derivative strain isolated from artificial water microcosm	This study
<i>P. fluorescens</i> 5EP126	<i>P. fluorescens</i> Pf0-1L(pCAR1::rfp) derivative strain isolated from artificial water microcosm	This study
<i>P. putida</i> KT2440	Sequenced strain	Bagdasarian <i>et al.</i> (1981); Nelson <i>et al.</i> (2002)
<i>P. putida</i> KT2440RG	Derivative strain of KT2440, spontaneous Rif ^r with introduced Gm ^r gene	Shintani <i>et al.</i> (2005a)
Plasmids or cosmids		
pBBR1MCS-5	Gm ^r , <i>lacZ</i> α mob; compatible with IncP, IncQ and IncW plasmids	Kovach <i>et al.</i> (1995)
pBBRantABC	pBBR1MCS-5 containing 8 kb <i>PstI</i> – <i>HindIII</i> from pSCos708	This study
pCAR1::rfp	pCAR1 containing <i>rfp</i> gene on 197 493 nt of pCAR1	Shintani <i>et al.</i> (2008a)
pCAR1::rfp_5EP124	pCAR1::rfp derivative plasmid of 5EP124	This study
pCAR1::rfp_5EP126	pCAR1::rfp derivative plasmid of 5EP126	This study
pCAR1::rfp_5EP83	pCAR1::rfp derivative plasmid of 5EP83	This study
pSCos708	Ap ^r , Km ^r , SuperCosI(Stratagene) with <i>EcoRI</i> insert of CA10 DNA	Nojiri <i>et al.</i> (2001)
pUC19	Ap ^r , <i>lacZ</i> α , pMB9 replicon	Sambrook & Russell (2001)
pUCantABC_5EP124	pUC19 containing 8 kb <i>PstI</i> – <i>HindIII</i> fragment on pCAR1::rfp_5EP124	This study
pUCantABC_5EP126	pUC19 containing 8 kb <i>PstI</i> – <i>HindIII</i> fragment on pCAR1::rfp_5EP126	This study
pUCantABC_5EP83	pUC19 containing 8 kb <i>PstI</i> – <i>HindIII</i> fragment on pCAR1::rfp_5EP83	This study
pUCantABC_Pf	pUC19 containing 8 kb <i>PstI</i> – <i>HindIII</i> fragment on pCAR1::rfp	This study
pUCFLG1	pUC19 containing 5.6 kb <i>EcoRI</i> – <i>SphI</i> fragment (fragment I in Fig. 5b) on pCAR1::rfp_5EP83 containing <i>antA</i> and partial region of ORF14	This study
pUCFLG2	pUC19 containing 3.7 kb <i>EcoRI</i> – <i>SphI</i> fragment (fragment II in Fig. 5b) on pCAR1::rfp_5EP83 containing partial regions of ORF24 and ORF9	This study

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 $\times$ 15 m, 0.25 μ m, GL Science). Column temperature was programmed to increase at a rate of 16 $^{\circ}$ C min⁻¹ from 80 $^{\circ}$ C (initial temperature) to 160 $^{\circ}$ C, and then at 60 $^{\circ}$ C min⁻¹ to 280 $^{\circ}$ 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 *m/z* 119 of the extract from each mutant to that of the 18 h sample of the parent strain Pf0-1L(pCAR1::rfp).

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 Pf0-1L(pCAR1::rfp). We selected three strains among the isolates from MRB2 (4ER211, 4ER212 and 4ER223) and MRC2 (5EP83, 5EP124 and 5EP126), and compared their abilities to degrade carbazole with that of the parent strain Pf0-1L(pCAR1::rfp).

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

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 *carAa* 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

Fig. 3. Growth curves of *P. fluorescens* Pf0-1L(pCAR1::rfp) and the mutants 5EP83, 5EP124 and 5EP126 in NMM-4 supplemented with carbazole. Three independent experiments showed similar results and only typical data are shown. Data represent the mean $\pm$ SD of technical triplicates.

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*

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* ($\Delta antA$) 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.

Fig. 5. (a) Southern hybridization analyses of total DNAs prepared from *Pf0-1L(pCAR1::rfp)* and 5EP83 using an *antA* probe (left) and an ORF9 probe (right). Each DNA was digested with *EcoRI* and/or *SphI*. Numbers indicate estimated band sizes (in kb). (b) Predicted DNA inversion in *pCAR1::rfp* that generated *pCAR1::rfp_5EP83* [based on the results of Southern hybridization analyses (a)]. Restriction sites are indicated by long (*EcoRI*) and short (*SphI*) vertical bars. Bidirectional black arrows indicate DNA fragments detected in Southern hybridization analyses (a). Conserved DNA regions are indicated by black/white triangles and the inverted DNA region by a dashed line. *antA* and ORF9 probes are indicated by parallel vertical lines. Disrupted *antA* ($\Delta antA$) on *pCAR1::rfp_5EP83* is shown by a pentagon with a dotted outline.

upstream region and a 3.5 kb *EcoRI*–*SphI* 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 *SphI*-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

Pf0-1 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 Pf0-1(pCAR1) in pure culture with carbazole as the sole carbon source (Takahashi *et al.*, 2009a). In the late stage of this pure culture, host Pf0-1 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 Pf0-1L(pCAR1::rff).

Considering that *antA*-ISPre1 (*tnpA2*) (7442–8871 nt, accession no. AB088420) and the reverse complement of ISPre1 (*tnpA1*-ORF9) (25 288–26 717 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 (pCAR1Δ1, pCAR1Δ3) (Urata *et al.*, 2004) and Pf0-1 (pCAR1Δ, pCAR1d, pCAR1SUCΔ1, pCAR1SUCΔ2, pCAR1SUCΔ3) (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 pCAR1Δ2 (lacks *antR*–*antABC* and *car*), previously found in Pf0-1(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 Pf0-1. While *antR* was missing from pCAR1d and pCAR1Δ2, 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 Pf0-1(pCAR1) to avoid excessive catechol (or brown pigment) accumulation. In contrast, systems

Fig. 6. Comparison of the genetic changes in pCAR1 in the host *P. fluorescens* Pf0-1. pCAR1d and pCAR1Δ2 were detected in individually cultured host strains (Takahashi *et al.*, 2009a). Black boxes with white pentagons indicate ISPre1 sequences, and black boxes with grey pentagons indicate ISPre2 sequences. White pentagons indicate the *repA* gene. Grey arrows indicate *antABC* and *car* operons. The inverted region in pCAR1 is highlighted by a bidirectional arrow.

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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REFERENCES

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (editors) (1990). *Current Protocols in Molecular Biology*. New York: Wiley.

- Bagdasarian, M., Lurz, R., Rückert, B., Franklin, F. C., Bagdasarian, M. M., Frey, J. & Timmis, K. N. (1981). Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. *Gene* **16**, 237–247.
- Compeau, G., Al-Achi, B. J., Platsouka, E. & Levy, S. B. (1988). Survival of rifampin-resistant mutants of *Pseudomonas fluorescens* and *Pseudomonas putida* in soil systems. *Appl Environ Microbiol* **54**, 2432–2438.
- Cowles, C. E., Nichols, N. N. & Harwood, C. S. (2000). BenR, a XylS homologue, regulates three different pathways of aromatic acid degradation in *Pseudomonas putida*. *J Bacteriol* **182**, 6339–6346.
- Harwood, C. S. & Parales, R. E. (1996). The β -ketoadipate pathway and the biology of self-identity. *Annu Rev Microbiol* **50**, 553–590.
- Heuer, H., Krsek, M., Baker, P., Smalla, K. & Wellington, E. M. (1997). Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Appl Environ Microbiol* **63**, 3233–3241.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., II & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175–176.
- Maeda, K., Nojiri, H., Shintani, M., Yoshida, T., Habe, H. & Omori, T. (2003). Complete nucleotide sequence of carbazole/dioxin-degrading plasmid pCAR1 in *Pseudomonas resinovorans* strain CA10 indicates its mosaicism and the presence of large catabolic transposon Tn4676. *J Mol Biol* **326**, 21–33.
- Miyakoshi, M., Urata, M., Habe, H., Omori, T., Yamane, H. & Nojiri, H. (2006). Differentiation of carbazole catabolic operons by replacement of the regulated promoter via transposition of an insertion sequence. *J Biol Chem* **281**, 8450–8457.
- Miyakoshi, M., Nishida, H., Shintani, M., Yamane, H. & Nojiri, H. (2009). High-resolution mapping of plasmid transcriptomes in different host bacteria. *BMC Genomics* **10**, 12.
- Nelson, K. E., Weinelt, C., Paulsen, I. T., Dodson, R. J., Hilbert, H., Martins dos Santos, V. A., Fouts, D. E., Gill, S. R., Pop, M. & other authors (2002). Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ Microbiol* **4**, 799–808.
- Nojiri, H. & Omori, T. (2007). Carbazole metabolism by *Pseudomonads*. In *Pseudomonas*, vol. 5, pp. 107–145. Edited by J.-L. Ramos & A. Filloux. New York: Springer.
- Nojiri, H., Sekiguchi, H., Maeda, K., Urata, M., Nakai, S., Yoshida, T., Habe, H. & Omori, T. (2001). Genetic characterization and evolutionary implications of a *car* gene cluster in the carbazole degrader *Pseudomonas* sp. strain CA10. *J Bacteriol* **183**, 3663–3679.
- Nojiri, H., Shintani, M. & Omori, T. (2004). Divergence of mobile genetic elements involved in the distribution of xenobiotic-catabolic capacity. *Appl Microbiol Biotechnol* **64**, 154–174.
- Nojiri, H., Sota, M. & Shintani, M. (2009). Catabolic plasmids involved in the degradation of polycyclic aromatic hydrocarbons and heteroaromatic compounds. In *Microbial Megaplasmids*, pp. 55–87. Edited by E. Schwarts. New York: Springer-Verlag.
- Ogawa, N., Chakrabarty, A. M. & Zaborina, O. (2004). Degradative plasmids. In *Plasmid Biology*, pp. 341–376. Edited by E. Funnell & G. J. Philips. Washington, DC: American Society for Microbiology.
- Ouchiya, N., Zhang, Y., Omori, T. & Kodama, T. (1993). Biodegradation of carbazole by *Pseudomonas* spp. CA06 and CA10. *Biosci Biotechnol Biochem* **57**, 455–460.
- Park, W., Jeon, C. O., Cadillo, H., DeRito, C. & Madsen, E. L. (2004). Survival of naphthalene-degrading *Pseudomonas putida* NCIB 9816-4 in naphthalene-amended soils: toxicity of naphthalene and its metabolites. *Appl Microbiol Biotechnol* **64**, 429–435.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Shintani, M., Habe, H., Tsuda, M., Omori, T., Yamane, H. & Nojiri, H. (2005a). Recipient range of IncP-7 conjugative plasmid pCAR2 from *Pseudomonas putida* HS01 is broader than from other *Pseudomonas* strains. *Biotechnol Lett* **27**, 1847–1853.
- Shintani, M., Yoshida, T., Habe, H., Omori, T. & Nojiri, H. (2005b). Large plasmid pCAR2 and class II transposon Tn4676 are functional mobile genetic elements to distribute the carbazole/dioxin-degradative *car* gene cluster in different bacteria. *Appl Microbiol Biotechnol* **67**, 370–382.
- Shintani, M., Yano, H., Habe, H., Omori, T., Yamane, H., Tsuda, M. & Nojiri, H. (2006). Characterization of the replication, maintenance, and transfer features of the IncP-7 plasmid pCAR1, which carries genes involved in carbazole and dioxin degradation. *Appl Environ Microbiol* **72**, 3206–3216.
- Shintani, M., Fukushima, N., Tezuka, M., Yamane, H. & Nojiri, H. (2008a). Conjugative transfer of the IncP-7 carbazole degradative plasmid, pCAR1, in river water samples. *Biotechnol Lett* **30**, 117–122.
- Shintani, M., Matsui, K., Takemura, T., Yamane, H. & Nojiri, H. (2008b). Behavior of the IncP-7 carbazole-degradative plasmid pCAR1 in artificial environmental samples. *Appl Microbiol Biotechnol* **80**, 485–497.
- Shintani, M., Takahashi, Y., Yamane, H. & Nojiri, H. (2010a). The behavior and significance of degradative plasmids belonging to Inc groups in *Pseudomonas* within natural environments and microcosms. *Microbes Environ* **25**, 253–265.
- Shintani, M., Yamane, H. & Nojiri, H. (2010b). Behavior of various hosts of the IncP-7 carbazole-degradative plasmid pCAR1 in artificial microcosms. *Biosci Biotechnol Biochem* **74**, 343–349.
- Silby, M. W., Cerdeño-Tárraga, A. M., Vernikos, G. S., Giddens, S. R., Jackson, R. W., Preston, G. M., Zhang, X. X., Moon, C. D., Gehrig, S. M. & other authors (2009). Genomic and genetic analyses of diversity and plant interactions of *Pseudomonas fluorescens*. *Genome Biol* **10**, R51.
- Simon, R., Priefer, U. & Pühler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Biotechnology* **1**, 784–791.
- Takahashi, Y., Shintani, M., Li, L., Yamane, H. & Nojiri, H. (2009a). Carbazole-degradative IncP-7 plasmid pCAR1.2 is structurally unstable in *Pseudomonas fluorescens* Pf0-1, which accumulates catechol, the intermediate of the carbazole degradation pathway. *Appl Environ Microbiol* **75**, 3920–3929.
- Takahashi, Y., Shintani, M., Yamane, H. & Nojiri, H. (2009b). The complete nucleotide sequence of pCAR2: pCAR2 and pCAR1 were structurally identical IncP-7 carbazole degradative plasmids. *Biosci Biotechnol Biochem* **73**, 744–746.
- Urata, M., Miyakoshi, M., Kai, S., Maeda, K., Habe, H., Omori, T., Yamane, H. & Nojiri, H. (2004). Transcriptional regulation of the *ant* operon, encoding two-component anthranilate 1,2-dioxygenase, on the carbazole-degradative plasmid pCAR1 of *Pseudomonas resinovorans* strain CA10. *J Bacteriol* **186**, 6815–6823.
- Versalovic, J., Schneider, M., de Bruijn, F. J. & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol Cell Biol* **5**, 25–40.
- Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.
- Williams, P. A., Jones, R. M. & Zylstra, G. (2004). Genomics of catabolic plasmids. In *Pseudomonas*, vol. 1, pp. 165–195. Edited by J. L. Ramos. New York: Kluwer Academic/Plenum Publishers.

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