Behavior of Various Hosts of the IncP-7 Carbazole-Degradative Plasmid pCAR1 in Artificial Microcosms

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pCAR1 endows carbazole-degrading ability on its host. Three different host Pseudomonas (P. fluorescens Pf0-1, P. resinovorans CA10dm4, and P. chlororaphis IAM1511) were prepared to compare their behaviors in artificial microcosms (sterile soil and water supplemented with carbazole) with previous results for P. putida SM1443 (Shintani et al., Appl. Microbiol. Biotechnol., 80, 485–497 (2008)). We monitored carbazole degradation, bacterial survival, and conjugative transfer of pCAR1. Carbazole degradations were not detected with any of three hosts in the soil microcosms, but were in most of the water microcosms. Degradative efficiency varied host by host. SM1443 is an appropriate host for carbazole degradation in soil microcosms, while CA10dm4 is for water microcosms. pCAR1 transfers were detected only in the water microcosms inoculated with SM1443 or IAM1511 as the host. This is the first report to compare the behaviors of various hosts of the same plasmid in these microcosms.

Key words: monitoring of degradative plasmid; IncP-7 plasmid pCAR1; carbazole

Degradative plasmids enable the host strain to degrade various xenobiotic compounds (toluene, xylene, naphthalene, and so on) and they sometimes transfer among different bacteria in the natural environment by conjugative transfer. There is now in-depth knowledge of the replication, maintenance, and conjugative transfer of incompatibility group P plasmids, including the IncP-1, IncP-7, and IncP-9 degradative plasmids. We have determined the nucleotide sequence of the IncP-7 carbazole degradable plasmid pCAR1, and have characterized its features including replication, partition, and conjugative transfer under laboratory conditions. Bioaugmentation is one of the methods of removing recalcitrant xenobiotic pollutants from contaminated sites by inoculating xenobiotic-degrading bacteria into the site. Unlimited transfer of the degradative plasmid among various bacteria in the natural environment is not necessarily appropriate in preventing the artificial spread of genes among unspecified bacteria. On the other hand, transfer of these plasmids possibly prevents loss of the degradative genes from the contaminated site once they have been transferred into indigenous bacteria. The behavior of such plasmids or hosts in nature is very important in assessing the risks and benefits of their use, but the knowledge of them is insufficient to predict the stability, transferability, or degradation ability of a degradative plasmid and its hosts in the environment. We have described a simple detection system for conjugative transfer using pCAR1 tagged with a transfer reporter gene encoding red fluorescent protein (RFP). We have also constructed artificial microcosms using sterile soil and river or pond water to simplify many factors that interact in a complex manner in nature rather than in natural environmental microcosms. In a previous study, we found as to the behaviors of P. putida SM1443 (derivative strains of P. putida KT2440) carrying pCAR1::rfp in artificial microcosms with high reproducibility, i) the high water contents in artificial soil microcosms cause carbazole degradation, ii) the frequency of pCAR1 transfer in the artificial soil microcosms is below the detection limit, iii) the presence of Ca$^{2+}$ and Mg$^{2+}$ is necessary for pCAR1 to transfer in artificial water microcosms. On the other hand, these behaviors of the plasmid and its host can change in case of using different host strains. For instance, we found a difference in host ability to metabolize carbazole as between P. putida KT2440(pCAR1) and P. fluorescens Pf0-1(pCAR1), and that the growth rate of Pf0-1(pCAR1) is much slower than that of KT2440(pCAR1). This difference in the growth rate is due to distinct regulation mechanisms of gene transcription encoding catechol degradation systems, catechol being an intermediate compound in the carbazole degradation pathway. In this study, we constructed three new host strains of pCAR1::rfp (P. fluorescens, P. resinovorans, and P. chlororaphis), and monitored their behaviors in artificial microcosms. A comparison of behavior among different host strains gave us insight into important aspects enabling us to predict their fates in the natural environment.

Materials and Methods

Bacterial strains, media, and culture conditions. The bacterial strains used in this study are listed in Table 1. Bacteria were grown in LB-medium (tryptone peptone 10 g l$^{-1}$, yeast extract 5 g l$^{-1}$, and NaCl 10 g l$^{-1}$) or on plates of nitrogen plus mineral medium-4 (NMM-4), with 0.1% (w v$^{-1}$) succinate, acetate, and citrate (SAC plates). The selection growth medium used to examine the carbazole metabolic capacity was NMM-4 supplemented with 0.1% (w v$^{-1}$) of carbazole as the sole source of carbon and energy (CAR plates), and kanamycin (Km, 50 μg ml$^{-1}$). Colonies on the CAR plates with a halo were...
counted as viable carbazole degraders. Plates were solidified with 1.6% (w/v) agar. The new hosts of pCAR1::rfp were constructed as follows: First, lacI gene was transposed from pBSL199lacI (transferred from E. coli S17-1) to each chromosome of Pseudomonas chlororaphis IAM1511, P. fluorescens P0-1, and P. resinovorans CA10dm4, with Tc-resistance as a selection marker. The resulting strains were renamed IAM1511L, Pf0-1L, and CA10L respectively. Then, pCAR1::rfp was transferred from SM1443(pCAR1::rfp) with Km-resistance as a selection marker. After confirmation of carbazole degradative ability and the transferability of the pCAR1 of each host strain, the new hosts were named IAM1511L(pCAR1::rfp), Pf0-1L(pCAR1::rfp), and CA10L(pCAR1::rfp) respectively.

Artificial microcosms. Artificial soils and water microcosms were prepared as described previously. In brief, artificial soil was mixed with 630 g of Sea Sand B (Nacalai Tesque, Kyoto, Japan), 175 g SiO\(_2\) (Kanto Chemical), and 20 g humic acids (Nacalai Tesque). After measurement of the maximum water-holding capacity, the water content of each artificial soil microcosm was supplemented with 0.01% (w/v) MRB and the MRC of this study MRB2 or MRC2. Each artificial soil microcosm was then inoculated with 630 g of Sea Sand B (Nacalai Tesque, Kyoto, Japan), 175 g SiO\(_2\), 20 g humic acids (Nacalai Tesque), 15) In brief, artificial soils were mixed vigorously using a direct mixer at maximum speed (AS 3000, As ONE, Osaka, Japan) for 10 min and then spread on SAC or CAR plates at appropriate dilution. Also, 100 µl of each artificial water microcosm was appropriately diluted and then spread on the plate. After incubation for 48–168 h at 30°C, the colonies on the plates were counted.

The remaining carbazole was measured by GC-MS analysis, as described previously. Carbazole was extracted from each artificial microcosm with ethyl acetate. GC-MS analysis was performed by the JMS-FTS system (JEOL Datum, Tokyo, Japan) and the capillary column InertCap500 (0.25 mm x 15 m, 0.25 µm, GL Science, Tokyo, Japan).

Plasmid transfer was detected as previously described. The rfp genes inserted in pCAR1 are repressed in the donor strain by the chromosomally introduced lacI gene product, and no fluorescence was detected. Only after conjugal transfer of the plasmid into the other strains, the red fluorescent phenotype was detected upon blue illumination (420–500 nm) when viewed through an amber filter (Dark Reader Transilluminator, BM Equipment, Tokyo, Japan).

To monitor changes in bacterial communities in the artificial environmental samples, terminal restriction fragment length polymorphism (tRFLP) analysis was performed as described previously with an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems).
Japan, Tokyo, Japan) with the size standard GeneScan-500 ROX (Applied Biosystems Japan). The results were analyzed with GeneScan® software ver. 1.0 (Applied Biosystems Japan).

**Standard DNA manipulations.** Total DNA was extracted from each bacterium, as described. DNA extraction from artificial environmental samples was performed with the PowerSoil® DNA kit (MO BIO Laboratories, Carlsbad, USA) or the NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The bacterial 16S rRNA gene was amplified using Takara ExTaq® polymerase (Takara Bio, Kyoto, Japan) with the 27F and 1378R primers (Sigma Genosys, Ishikari, Japan) according to the manufacturer's instructions. The PCR conditions were 96°C for 2 min, followed by 30 cycles of 96°C for 30 s, 60°C for 60 s, and 72°C for 1 min. RFLP analysis was performed by digesting the above 16S rRNA gene with MnlI (New England BioLabs, Ipswich, USA) and HaeII (Takara Bio) for 3 h at 37°C, followed by separation by 2% agarose gel electrophoresis.

**Results**

**Behaviors of three distinct host strains of pCAR1 in the artificial soil microcosms**

We have reported that the water content of the soil influenced the degradative ability of carbazole when *P. putida* SM1443 (a *P. putida* KT2440 derivative strain) was inoculated as a carbazole-degrading host of pCAR1 (shown in Fig. 1A as reference). In this study, we prepared three other host strains, *P. fluorescens* PF0-1L, *P. chlororaphis* IAM1511L, and *P. resinovorans* CA10L. After inoculation into the same artificial soil microcosms of each of them, we assessed whether the water contents affected carbazole degradation.

*P. fluorescens* PF0-1L(pCAR1::rfp) (Fig. 1B)

The viable bacterial number decreased in each microcosm (from $10^8$ to $10^7$ CFU/g), and the carbazole degraders were not detectable from just after inoculation to the end of monitoring for each microcosm (below 10 CFU/g). We did not detect any transconjugants. No carbazole degradation was detected in the three microcosms, suggesting that this host strain is not appropriate for the degradation of carbazole in the soil environments. As for the change in the bacterial community, the ratio of *P. putida* and *C. testosteroni* increased similarly in MSA and MSB, whereas that of MSC did not change, and those of *S. meliloti* or *P. chlororaphis* increased.

*P. resinovorans* CA10L(pCAR1::rfp) (Fig. 1C)

In the microcosms inoculated with CA10L(pCAR1::rfp), the viable bacterial number decreased from $10^7$ to $10^5$ CFU/g to $10^3$–$10^7$ CFU/g in MSA, MSB, and MSC. The number of carbazole degraders was detected only just after inoculation ($10^5$ CFU/g), but that at 5–30 d after inoculation was below the detection limit ($<10^5$ CFU/g) for each microcosm. We did not find any transconjugants, and carbazole degradation was not detected, either. As for the bacterial community changes in MSB and MSC, results similar to those for the samples inoculated with PF0-1L(pCAR1::rfp) were obtained, while those in MSA were different from the case of PF0-1L(pCAR1::rfp). The ratio of *P. putida* and *C. testosteroni* did not change, and that of *P. chlororaphis* increased in the case of CA10L(pCAR1::rfp).

*P. chlororaphis* IAM1511L(pCAR1::rfp) (Fig. 1D)

The viable bacterial number of the microcosms with inoculation of IAM1511L also decreased from $10^8$ to $10^6$ CFU/g over 30 d, and the number of carbazole degraders decreased from $10^9$ to about $10^5$ CFU/g in MSA, MSB, and MSC. The decrease rate of the degraders was slower than that of the microcosms inoculated with CA10L(pCAR1::rfp), but no transconjugants and no carbazole degradation was detected in the three microcosms. We did not find any dramatic change in the bacterial community in the MSB, whereas the ratio of *P. putida* (KT2440 and D51) and *C. testosteroni* IAM12419 increased in the MSA and MSC.

**Behaviors of each host strain in the artificial water microcosms**

Three kinds of artificial water microcosms were prepared: MRA (buffer), MRB2 (filtered Arakawa River water), and MRC2 (filtered Shinobazu Pond water), as previously described. We compared the results for them with those of *P. putida* SM1443(pCAR1::rfp) as well as the artificial soil microcosms (shown in Fig. 2A as reference).

*P. fluorescens* PF0-1L(pCAR1::rfp) (Fig. 2B)

As a result of inoculation with PF0-1L(pCAR1::rfp), the viable bacterial number in MRA decreased from $10^7$ to $10^5$ CFU/ml, whereas those in MRB and MRC2 decreased slightly, from $10^5$ to $10^4$ CFU/ml. The numbers of carbazole degraders decreased just after inoculation of the donors. However, in the cases of MRB2 and MRC2, we detected increases in the degraders after 15 d, and also found degradation of carbazole. No transconjugants were detected in the three microcosms. Forty-two after inoculation of the donor, 6% and 32% of the carbazole remained in MRB2 and MRC2, whereas nearly 100% of the carbazole was still found in MRA. As for the change in the bacterial community, the ratio of *S. paucimobilis* increased in MRA but not in MRB2 or MRC2. Note that the ratio of *P. resinovorans* increased in MRC2, whereas those in MRA decreased.

*P. resinovorans* CA10L(pCAR1::rfp) (Fig. 2C)

In the water microcosms inoculated with *P. resinovorans* CA10L(pCAR1::rfp), viable bacterial numbers were at the same levels 30 d after inoculation. The numbers of carbazole-degrading bacteria increased from $10^7$ to $10^5$ CFU/ml at 5–10 d after inoculation in each microcosm. No transconjugants were detected in them. Twenty-eight d after inoculation of the donor, 19%, 1.4%, and below the detection limit ($<0.001$%) of the carbazole remained in MRA, MRB2, and MRC2 respectively. The change in the bacterial community was similar in MRB2 and MRC2, whereas that in MRA was different, as in the ratios of *P. alcaligenes*, *P. fluorescens*, and *P. putida*.

*P. chlororaphis* IAM1511L(pCAR1::rfp) (Fig. 2D)

As for the microcosms inoculated with IAM1511L(pCAR1::rfp), the number of viable bacteria was at a similar level from the beginning to the end of monitoring. The number of carbazole degraders increased ($10^7$ to $10^5$ CFU/ml) in MRA, while those in MRB2 and MRC2 decreased to $10^5$–$10^4$ CFU/ml after 5 d, and then increased to $10^5$–$10^4$ CFU/ml. Carbazole remaining at 30 d after inoculation were 25%, 14%, and 16% in MRA, MRB2, and MRC2 respectively. We found only one colony of the transconjugant from MRC2, which corresponded to $10^5$ CFU/ml transconjugants. RFLP analysis and PCR
were performed to determine the genus and species of the transconjugant, and we assessed whether this strain had pCAR1 or the lacI gene on the chromosome. The transconjugant was *P. resinovorans* as well as the sample with inoculation of SM1443(pCAR1::rfp) \(^{(15)}\) (data not shown). As for the change in the bacterial community, the ratio of *P. resinovorans* increased in MRB2 and MRC2, whereas those in MRA did not change.

**Discussion**

In this study, we inoculated each of three host strains of pCAR1 (*P. fluorescens*, *P. resinovorans*, and...
Changes in Carbazole Remaining and Bacterial Numbers in the Artificial Water Microcosms.

Results for the various hosts are shown in (A) SM1443(pCAR1::rfp), reference data, (B) Pf0-1L(pCAR1::rfp), (C) CA10L(pCAR1::rfp), and (D) IAM1511L::pCAR1::rfp). In the (A) to (D) panels, MRA, MRB2, and MRC2 are the artificial water microcosms containing carbon-free buffer, filtered river water, and filtered pond water respectively. Symbols are as described in the Fig. 1 legend. Each data point for carbazole remaining (top) and changes in the bacterial numbers (middle) represents the mean ± standard deviation of three replicates, and the y-axis of each graph is logarithmic.

*P. chlororaphis* into artificial microcosms and monitored their behaviors. Note that the degradation rates of these strains and SM1443(pCAR1) in a laboratory environment (NMM-4 liquid medium with carba-zole as sole carbon source) were different: SM1443, IAM1511L, and CA10L with pCAR1 degraded carba-zole about 24h after the inoculations, whereas Pf0-1L with pCAR1 degrade more than 96h afterwards (data not shown). As for the artificial soil microcosms, no carba-zole degradation was detected regardless of the water content (MSA to MSC) or kind of host strain at 30d after inoculation (Fig. 1). The reason we did not detect carba-zole degradation in the soil microcosms inoculated with three hosts is clearly that the number of carba-zole-degrading bacteria decreased to below 10 CFU/g in all samples (Fig. 1). Especially, we did not detect any degraders just after the inoculation of Pf0-1L(pCAR1) were unable to metabolize catechol, the
intermediate compound of the carbazole degradation pathway, and accumulation of catechol is toxic to the cells). (ii) They may have been alive and may have adhered to the particles in the soil microcosms, as is confirmed by the fact that Pfl-1 was isolated as a strain adhesive to the soil particles. Carbazole degraders in the other soil microcosms inoculated with CA10L(pCAR1::rfp) or IAM1511L(pCAR1::rfp) decreased similarly, although the rates of the latter were slower than those of the former (Fig. 1C, D). Currently, we cannot explain the differences in survival ability between SM1443 and the other three host strains, but we have concluded that SM1443(pCAR1::rfp) was the most suitable host strain for degradation among four hosts in the soil environment.

As for behavior in the artificial water microcosms, carbazole degradation occurred differently in the various microcosms inoculated with various hosts of pCAR1. The ratio of carbazole degradation in the MRA (buffer) inoculated with the three hosts individually was lower than that of SM1443(pCAR1::rfp) (Fig. 2). Considering that Pfl-1L(pCAR1::rfp) did not degrade carbazole effectively, it is not surprising that the carbazole degraders decreased and that we did not detect carbazole degradation in MRA (Fig. 2B). As for the samples with CA10L(pCAR1::rfp) and IAM1511L(pCAR1::rfp), the numbers of carbazole degraders increased after inoculation, although the degraders in the MRB2 and MRC2 inoculated with IAM1511L(pCAR1::rfp) decreased once (Fig. 2C, D). Thus, the carbazole degradation may have proceeded after a longer term of incubation. Surprisingly, carbazole degradations were found in the Pfl-1L(pCAR1::rfp) inoculated samples, and the numbers of carbazole degraders of them fell once and then rose again (Fig. 2B). These phenomena were found reductively, and carbazole degradation occurred together with regaining of the number of carbazole degraders. RFLP comparisons showed that the “reappeared” degraders were Pfl-1L(pCAR1::rfp) (data not shown). Similar results were also found in the samples with IAM1511L(pCAR1::rfp), and now we are analyzing these strains genetically in more detail.

We have found that Ca^{2+} and Mg^{2+} are important to the occurrence of transconjugants when SM1443(pCAR1::rfp) is inoculated in the artificial water microcosms. Considering the composition of the MRB2 and MRC2 used in this study, the concentration of these ions may have been enough to cause transconjugants, but we found transconjugants only in the MRC2 inoculated with IAM1511L(pCAR1::rfp) (Fig. 2D). The transconjugants obtained were P. resinovorans in both SM1443(pCAR1::rfp) and IAM1511L(pCAR1::rfp), suggesting that P. resinovorans is a good recipient of pCAR1 in the environment that we used. Surprisingly, we did not detect any transconjugants from MRB2 or MRC2 with CA10L(pCAR1::rfp), although the frequency of pCAR1 transfer from P. resinovorans to P. resinovorans was higher than those from P. chlororaphis or P. putida to P. resinovorans. The stability of the plasmid in the different hosts could have altered, however; pCAR1 was stably maintained in Pseudomonas bacteria, at least under laboratory conditions. In addition, the efficiency of carbazole degradation in MRB2 and MRC2 with CA10L(pCAR1::rfp) was higher than with the others, whereas initial degradation was also efficient in the microcosms with SM1443(pCAR1::rfp) (Fig. 2). This indicates that P. resinovorans may have been appropriate degraders in these environments. The reason only P. resinovorans was obtained as a transconjugant is probably it was not only a good recipient of pCAR1 but also a good degrader of carbazole in the artificial microcosms.

To the best of our knowledge, this is the first report to show the different behaviors (degrading ability, survival, and conjugative transfer) of plural hosts that possess the same degradative plasmid in artificial microcosms. Our results indicate the importance of host selection of degradative plasmid (or genes) in making use of them in bioaugmentation. Validation of the various conclusions of this study in natural samples, such as unsterilized soil, sand, and river water, should prove useful in utilizing the plasmid host strains in various applications, including bioremediation.

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