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Fusion and Compatibility of Camphor and Octane Plasmids in *Pseudomonas*

(plasmid transduction/recombination)

GEORGE I. N. CHOU*, DVORAH KATZ, AND I. C. GUNSALUS

Biochemistry Department, University of Illinois, Urbana, Ill. 61801

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ABSTRACT **The octane (OCT) plasmid in *Pseudomonas putida* derived from the ω-hydroxylase-carrying strain of Coon and coworkers is transferable to the camphor (CAM) plasmid-bearing strain by conjugation or by transduction. While the majority of the Cam$^+$Oct$^+$ exconjugants segregate Cam$^+$ or Oct$^+$ cells, exconjugants with stable Cam$^+$Oct$^+$ phenotype (CAM–OCT) can be detected at a low frequency. The transductants are all of the CAM–OCT phenotype. In the stable Cam$^+$Oct$^+$ strains, the OCT plasmid resembles the CAM plasmid with respect to curing by mitomycin C, transfer in conjugation, and reaction to *ts* (temperature-sensitive) mutation specifically affecting CAM plasmid replication. Therefore, it is suggested that certain regions of homology exist between the CAM and OCT plasmids that enable them to recombine to form a single plasmid, and to overcome the incompatibility barrier that prevents their coexisting.**

Plasmids coding for the early steps of peripheral metabolic pathways and their conjugal transfer have been increasingly documented in fluorescent pseudomonads. Such are the gene clusters of camphor-degrading enzymes (1, 2) and the enzymes degrading octane (3), salicylate (4), and naphthalene (5). It appears reasonable to suggest that plasmids might have played an important genetic role in the development of the unusual metabolic diversity among the *Pseudomonas* species (6). The self-transmissible properties of plasmids enables us to construct cells that harbor more than one plasmid and to study their relationship. We have studied particularly in this connection the octane (OCT) and the camphor (CAM) metabolic plasmids. The CAM plasmid was found in a *Pseudomonas putida* strain PpGl (7) and the OCT plasmid in a *P. putida* ATCC 17633 (PpG6) formerly termed *P. oleovorans*, isolated by Coon and coworkers (8). The lack of a known genetic system in the latter strain and its resistance to the phage pf16 precluded an easy genetic analysis in this strain. Therefore, the OCT plasmid was transferred into *P. putida* recipient strain where genetic analysis by transduction is possible. Our earlier paper (3) described the conjugal transfer of the OCT plasmid and the incompatibility of the CAM and the OCT plasmids; the present paper describes a genetic situation in which the CAM and the OCT plasmids do coexist. Chakrabarty, using a different approach (9), has increased the frequency of the CAM–OCT cotransfer in conjugal transfer.

Abbreviations: Phenotypes, genotypes, and plasmids, respectively, of cells that can degrade the following compounds are: camphor: Cam, *cam*, CAM; octane: Oct, *oct*, OCT; naphthalene: Nah, *nah*, NAH. CAM–OCT refers to a fused CAM and OCT plasmid.

* Present address: Infectious Disease Unit, Massachusetts General Hospital, Boston, Mass. 02114.

METHODS

Organisms and Media. Three separate isolates of wild-type *Pseudomonas putida* and their derivatives used in this study are listed in Table 1. PpG numbers indicate genus—*Pseudomonas*, species—*putida*, G—Gunsalus collection. L-broth (10) served as a complete medium, and minimal salt medium (11) was used with the addition of 5 or 10 mM of the appropriate carbon source. Application of D(+)-camphor and *n*-octane (Phillips Petroleum Co.) has been described elsewhere (2, 3). Unless mentioned otherwise, growth was at 30°.

Conjugation: Plate Mating. Both the donor and the recipient cells were grown to stationary phase at 30° in complete medium. The donor cells were diluted 1/10 and 1/100 in saline and mixed carefully with the same volume of undiluted culture of the recipient strain. Samples of 0.1 ml were plated on a selective medium that permitted growth of the exconjugants only. The reversion frequency of the strains used in these experiments was $\leq 10^{-9}$.

Transduction. Phage pf16 was used as the transducing phage as described (12).

Mitomycin C Curing was according to Rheinwald *et al.* (2).

Selection of ts(Cam) Mutants. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was used for mutagenesis (2). *ts*(Cam) mutants were selected as those that could not utilize D(+)-camphor as a sole carbon source at 37° but could use it at 24°. Among *ts*(Cam) mutants, those that irreversibly lost the Cam$^+$ phenotype when grown in L-broth at 37° were identified as mutants in which the CAM replication mechanism has been damaged. We used the two methods to identify the mutation as a plasmid or chromosomal: a conjugal transfer of the plasmid from the mutant into wild-type recipient, or infection of the cured mutant by wild-type plasmid. The Cam$^+$ phenotype of the exconjugants was then tested at 24° and 37°.

RESULTS

The conjugal transfer of the OCT plasmid from *P. oleovorans* strain PpG969 to *P. putida* occurs at a low frequency ($<1 \times 10^{-9}$) (refs. 3 and 9; see Table 2). When CAM cured (CAM^d) *P. putida* strains (PpG572, PpG584, and PpG277) were used as the recipient strain, stable Oct$^+$ exconjugants were recovered. These *P. putida* Oct$^+$ exconjugants resembled the *P. oleovorans* Oct$^+$ in their donor ability for OCT but were resistant to the curing effect of mitomycin C. In some cases, exconjugants of an exceptionally good donor ability were isolated. One example is PpG972, an effective donor for OCT plasmid that could mobilize chromosomal markers efficiently.

TABLE 1. *Pseudomonas strains and their origin*

PpG	Genotype*	Phenotype	Parent†	Treated‡	Ref.
6	*wt*/OCT	Oct$^+$	—	Octane enrichment	8
968	*wt*/OCT^d	Oct$^-$	6	MC	
969	*his*-901/OCT	His$^-$Oct$^+$	6	NG	
7	*wt*/NAH	Nah$^+$	—	Naphthalene enrichment	5
377	*wt*/NAH^d	Nah$^-$	7	S	5
378	*leu*-801/NAH	Leu$^-$Nah$^+$	7	NG	5
379§	*leu*-801/NAH	Leu$^-$Nah$^+$	378	NG	5
1¶	*wt*/CAM	Cam$^+$	—	Camphor enrichment	7
572	*wt*/CAM^d	Cam$^-$	1	PC	2
574	*wt*/CAM^d	Cam$^-$	1	PC	2
584	*str*-600/CAM^d	Str^{r}Cam$^-$	574	S	
273§	*trpB*615/CAM	Trp$^-$Cam$^+$	1	PC	2
277§	*trpB*615/CAM^d	Trp$^-$Cam$^-$	273	MC	
970§	*trpB*615/CAM-OCT	Trp$^-$Cam$^+$Oct$^+$	969 × 273	C, aux	
972	*trpB*615/CAM^{d}OCT	Trp$^-$Cam$^-$Oct$^+$	969 × 277	C, aux	
981	*str*-600/CAM^{d}OCT	Str^{r}Cam$^-$Oct$^+$	969 × 584	C, aux	
982	*str*-600/CAM^{d}OCT	Str^{r}Cam$^-$Oct$^+$	969 × 584	C, aux	
977§	*trpB*615/CAM-OCT	Trp$^-$Cam$^+$Oct$^+$	981 → 273	*T*(pf16)	
978	*wt*/CAM^{d}OCT	Cam$^-$Oct$^+$	981 → 572	T(pf16)	
390	*wt*/CAM^{d}NAH	Cam$^-$Nah$^+$	379 × 572	C, aux	
*ts*230	*ts*230(Cam)*trpB*615/CAM	Trp$^-$Cam^{ts}	273	NG	
*ts*230-1	*ts*230(Cam)/CAM	Cam^{ts}	572 → *ts*230	T(pf16)	
*ts*230-2	*ts*230(Cam)*trpB*615/CAM^d	Trp$^-$/CAM^d	*ts*230	MC	
*ts*230-3	*ts*230(Cam)/CAM^d	Cam$^-$	*ts*230-1	MC	
*ts*230-4	*ts*230(Cam)*trpB*615/CAM^{d}OCT	Trp$^-$Cam$^-$Oct$^+$	*ts*230 = 3 × 972	C,aux	
*ts*230-5	*ts*230(Cam)*trpB*615/CAM-OCT	Trp(Cam$^+$Oct$^+$)^{ts}	972 → *ts*230	T(pf16)	
*ts*230-6	*ts*230(Cam)*trpB*615/CAM-OCT	Trp(Cam$^+$Oct$^+$)^{ts}	981 → *ts*230	T(pf16)	

* Chromosomal loci precede, plasmid follows. ^{d}, cured strains; *wt*, prototroph; *his*, histidine; *leu*, leucine; *str*, streptomycin; *trp*, tryptophan; *ts*, temperature-sensitive.

† ×, conjugation; →, transduction; donor first followed by recipient (D×R or D→R).

‡ S, spontaneous; MC, mitomycin C; NG, nitrosoguanidine; PC, penicillin–cycloserine; C,aux, counter selection against auxotrophs; T, transduction.

§ In conjugation high-frequency transfer plasmids.

¶ For point mutants in camphor plasmid, see ref. 2.

In the conjugation between *P. oleovorans* and *P. putida* Cam$^+$ (PpG1 or PpG273), the exconjugants were unstable and eventually segregated completely to Cam$^+$ or Oct$^+$ (Table 3). This incompatibility between the CAM and OCT plasmids was not due to surface exclusion but rather to deletion of one of the plasmids. The naphthalene (NAH) plasmid, however, was found to be compatible with the OCT. As a very rare event, a cell line of a stable Cam$^+$Oct$^+$ phenotype arose spontaneously, such as strain PpG970. In this strain, designated CAM–OCT, the plasmids behave as if they were fused; they cotransfer in conjugation and are cocured by mitomycin C (Table 4). When a selective pressure aimed for Cam$^+$ or Oct$^+$ was applied in the presence of mitomycin C, 100% of the survivors were Cam$^+$Oct$^+$ (Table 4*B*). Chakrabarty (9) was able to increase the formation of CAM–OCT exconjugants to some extent by UV irradiation.

As an alternative to conjugal transfer, we used the transduction system of *P. putida* by way of the transducing phage pf16 (13). The donor strain in the transduction was Oct$^+$

TABLE 2. *Transmissibility of OCT plasmid to P. putida strains*

Donor	Recipient	Mode*	Frequency† of transfer
PpG969	PpG 1	Conjugation	10^{-9}
PpG969	273	Conjugation	10^{-9}
PpG969	277	Conjugation	10^{-9}
PpG969	572	Conjugation	10^{-9}
PpG969	584	Conjugation	10^{-9}
PpG972	572	Conjugation	8×10^{-2}
PpG972	1	Conjugation	3×10^{-2}
PpG981	1	Transduction	4×10^{-8}
PpG981	273	Transduction	5×10^{-8}

* For conjugation procedure, see *Methods*. For transduction, phage lysate was used at the multiplicity of infection of 0.2.

† Frequency was defined as the number of conjugants (or transductants) per donor cell (or donor phage).

TABLE 3. *Incompatibility of CAM, OCT, and NAH plasmids*

Donor	Recipient	Selection	Exconjugants* Total	Exconjugants* NSM/Sel.†
273/CAM	981/OCT	Cam$^+$	42	0.24
273/CAM	982/OCT	Cam$^+$	14	0.14
969/OCT	273/CAM	Oct$^+$	7	0.43
379/NAH	982/OCT	Nah$^+$	20	1.0

* Exconjugants were patch-tested on selection plates. Single cell colonies were selected by streaking on L-plates followed by phenotype scoring on the appropriate plates.

† *NSM*, nonselected marker; *Sel.*, selected marker.

and the recipient strain Oct$^-$. We observed that the yield of Oct$^+$ transductants was dependent on the presence of the CAM plasmid in the recipient. The number of Oct$^+$ transductants was 100 times greater when the recipient strain in the transduction harbored the CAM plasmid (Table 5). Nonfunctional CAM plasmids (which resulted from point mutation; ref. 2) were found to be as good as the native plasmids in stimulating the formation of Oct$^+$ transductants. As a result, all the transductants were of a stable Cam$^+$Oct$^+$ phenotype. These plasmids cotransferred, were cocured by mitomycin C, and were therefore designated CAM–OCT like those that were formed by conjugation.

These observations led us to believe that CAM–OCT is a fused plasmid. Therefore, we isolated a mutant, *ts*230, that replicated the CAM plasmid at 24° but lost it during growth in L-broth at 37°. The mutation was located on the bacterial chromosome and was specific for CAM replication. When the *ts*230 mutant was cured of its own CAM plasmid by mitomycin C or exposure to growth at the high temperature, and then reinfected by the CAM plasmid of PpG273, the cells kept their temperature sensitivity for CAM replication. On the other hand, when the cured *ts*230 was reinfected by OCT plasmid, it did replicate the OCT plasmid at the high temperature. When *ts*230/CAM was used as a recipient in Oct$^+$ transduction, and the CAM–OCT transductants were tested as to the effect of high temperature on the maintenance of Cam$^+$ and Oct$^+$, it was found that they always behaved as if they were a single replicon (Fig. 1).

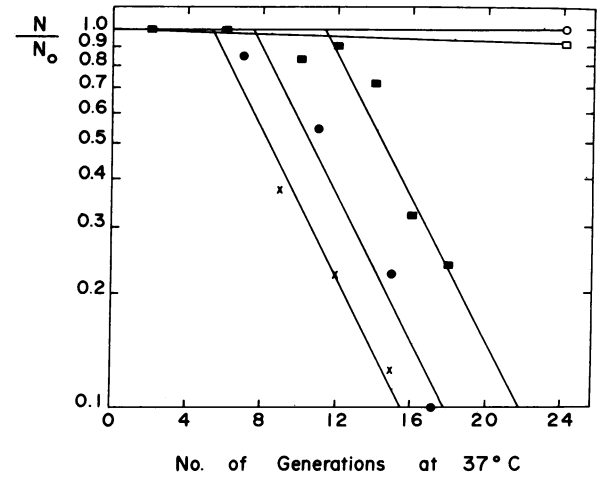

FIG. 1. The effect of the *ts*230 mutation on the maintenance of the CAM, OCT, and CAM–OCT plasmids. *ts*230 strains harboring the different types of plasmids were grown for various lengths of times at 37°. Samples were plated on complete medium at 24°, and single cell colonies were tested for their plasmid phenotype at 24°. N/N_0, fraction in the population of Cam$^+$ or Oct$^+$ or Cam$^+$Oct$^+$, according to the phenotype of the strain. 24°: (□) *ts*230 (CAM), *ts*230-5 (CAM–OCT), *ts*230-6 (CAM–OCT). 37°: (■) *ts*230 (CAM), (●) *ts*230-5 (CAM–OCT), (×) *ts*230-6 (CAM–OCT). 24° or 37°: (○) PpG273 (CAM) or (CAM–OCT), *ts*230-4 (OCT).

DISCUSSION

OCT and CAM plasmids are incompatible in *P. putida* (3). Little is known about the mechanism that leads to incompatibility of plasmids, but we assume that the phenomenon indicates a close relationship between them (14). The CAM–OCT system appears to provide an example where a close relationship (probably in terms of homologous regions) permits recombination between the plasmids.

TABLE 4. *Properties of PpG970 CAM–OCT*

A. Conjugal cotransfer of the CAM and the OCT plasmids

Donor	Recipient	Exconjugant Phenotype	Exconjugant Frequency
970/CAM-OCT	572 *wt*/CAM^d	Cam$^+$Oct$^+$	10^{-1}
	584 *str*-600/CAM^d	Cam$^+$Oct$^+$	10^{-1}
	968 *wt*/OCT^d	Cam$^+$Oct$^+$	10^{-1}
	7 *wt*/NAH	Cam$^+$Oct$^+$	10^{-1}
	377 *wt*/NAH^d	Cam$^+$Oct$^+$	10^{-1}

B. Segregation and curing of the CAM and the OCT Plasmid in PpG970

Treatment	Cured cell*/Total
100 generations in L-broth	0/1790
200 generations in L-broth	0/3860
20 μg of mitomycin C in L-broth	388/415
20 μg of mitomycin C in minimal medium + tryptophan + D(+)-camphor	0/575
20 μg of mitomycin C in minimal medium + tryptophan + *n*-octane	0/600

* All cured cells were CAM^d and OCT^d.

Conjugal transfer and transduction were used to introduce the CAM and OCT plasmids into the same cell. By the former technique, a very low frequency of CAM–OCT (stable Cam$^+$ Oct$^+$) plasmids was obtained. In the transduction experiments, all the transductants were of the CAM–OCT phenotype, and the Cam$^+$ and Oct$^+$ phenotypes were cotransferred in conjugation and were also cured simultaneously. These results suggest that recombination between two complete plasmids, which is an inefficient and rare event, is enhanced when a linear piece of DNA (carried by the transducing phage pf16) is present instead of a full plasmid. The nature of the new relationship between the CAM and OCT plasmids may be explained by the assumption that they fused to become one plasmid using a single replication system.

Using the temperature-sensitive mutant *ts*230, a chromosomal mutation in which CAM replication was specifically

TABLE 5. *Effect of CAM plasmid in recipient on octane plasmid gene transduction**

Recipient (PpG no.)†	Genotype	Oct$^+$/10^9 donor cells
1	*wt*/CAM	25
572	*wt*/CAM^d	0.3
273	*trp*B615/CAM	35
545	*wt*/CAM*cam102*‡	5
543	*wt*/CAM*cam100*	31
553	*wt*/CAM*camD121*	43
557	*wt*/CAM*camG133*	31
563	*wt*/CAM*cam200*	75

* Transducing phage pf16 was grown on strain 981, multiplicity of infection 0.2; selection for Oct$^+$.

† Donor 981 = *str*-600/CAM^{d}OCT.

‡ CAM plasmid point mutants. See Rheinwald *et al.* (2).

affected, we were able to support this hypothesis. Other similar chromosomal genes in another plasmid system have been described (15, 16). As a result of the mutation, the CAM plasmid is diluted out when cells are grown at 37°. It is difficult to say whether the slow rate of the loss of the CAM plasmid at 37° reflects the number of plasmid copies existing in the cells, or an incomplete block of the plasmid replication. When the *ts*230 strain harbors the OCT plasmid, it can support plasmid replication at 37° as well as at 24°. However, in CAM–OCT cells both phenotypes, the Cam^+ and the Oct^+, were eliminated when grown at 37°. The rate of elimination was the same as for the CAM plasmid alone, although the CAM–OCT plasmid started disappearing somewhat earlier. Thus, it seems clear that the CAM–OCT plasmid uses the CAM replication machinery, and that it contains as an integral part a piece of the OCT plasmid.

The OCT plasmid is probably composed of at least two parts: the genes that specify the enzymes needed for *n*-octane degradation (Oct), and the genes coding for its autonomous replication (17). Transduction of the OCT plasmid can include both parts, but this occurs at a very low frequency (3×10^{-10}). Enhancement in the frequency of the formation of the Oct^+ transductants (3×10^{-8}) by the CAM plasmid is possible when it is present in the recipient cell. We interpreted this by the assumption that the Oct portion of the OCT plasmid was transduced separately, and only if it has been incorporated into an existing replicon (CAM) in the recipient strain will its function be expressed.

The ability of plasmids to fuse and to disintegrate is known in various R-factor systems (18, 19), where these processes are controlled by the bacterial host and are influenced by growth conditions (19). R-factors were also found to be distributed among various bacterial strains by means of transduction (20). However, in most of the cases, the transduced plasmid was recovered as an autonomous replicating unit. In the case of the R213 factor, the plasmid was found to be too large to be transduced as one unit, and could only be recovered among the transductants if a homologous plasmid were present in the recipient strain (21).

The fusion of the CAM and OCT plasmids is somewhat different because two incompatible plasmids fuse here, while the common reaction between these plasmids is to segregate when they are forced to exist in the same cell; very rarely do they recombine. Recombination between these plasmids is greatly enhanced when the OCT plasmid is transduced into a CAM-harboring strain. This observation indicates that regions of homology exist between the two plasmids. Although the two plasmids were isolated independently, and found in different wild-type strains using different enrichment systems (6, 8), a region of homology could have originated from the same defective phage (22, 23). Such a common base sequence could resist major evolutionary changes because of its essentiality to the survival of the plasmid.

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