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Dissociation of a Degradative Plasmid Aggregate in *Pseudomonas*

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The infectious plasmid OCT, which specifies a set of dissimilatory enzymes responsible for the degradation of *n*-octane, has been shown to be an aggregate of a noninfectious OCT plasmid and an infectious plasmid with sex factor activity. The infectious plasmid, which can be eliminated from the cells of *Pseudomonas putida* by mitomycin C treatment without loss of the OCT plasmid and vice versa, has been designated as factor K. The infectious plasmid (factor K) is not only responsible for the mobilization of OCT, but also mobilizes chromosomal genes at a frequency of 10^{-2} to 10^{-3} per donor cell. Whereas OCT is incompatible with another degradative plasmid, CAM, factor K appears to be compatible with it.

We have shown recently that the genes coding for octane dissimilatory enzymes in *Pseudomonas oleovorans* are borne on a plasmid (OCT plasmid) and can be transferred from *P. oleovorans* to a number of other *Pseudomonas* species (4, 5). Plasmids like OCT, CAM, and SAL, which specify a complete degradative pathway that converts a complex organic compound (for octane, camphor, and salicylate, respectively) to a simpler metabolite, have been designated as "degradative plasmids" because of their essentiality for such conversion (4). It must be stressed that the evidence for the extrachromosomal nature of such genetic entities specifying the degradative pathways is wholly genetic, and it has not been possible to isolate any of these plasmids physically separate from the chromosome either by cesium chloride or by cesium chloride-ethidium bromide density gradient centrifugation (3). We have shown previously that the degradative plasmids CAM and OCT are incompatible with each other and cannot coexist in the same *P. oleovorans* cell. This report concerns the interaction of these two plasmids in *P. putida* and the further resolution of the OCT plasmid into its component, such as the infectious plasmid (herein designated as factor K) and a noninfectious OCT plasmid. The role of factor K in the mobilization of other noninfectious plasmids will be the subject of a future communication.

MATERIALS AND METHODS

Bacterial strains and their propagation. Most of the methods enumerating the cultural conditions and growth characteristics of the strains have been described previously (3, 5, 10). The relevant properties of the strains are given in Table 1. Selection of OCT^+ exconjugants is done routinely on plates containing a mixture of octane and decane (1:1) as the sole carbon source.

Curing and transfer of plasmids. The detailed methods for curing the plasmids have already been published (3, 5). The optimal conditions for transfer of the OCT plasmid have also been described (5). Since OCT^+ *P. putida* PpG1 exconjugants grow rather slowly with octane (or an octane-decane mixture), the plates are usually incubated for 4 or 5 days at 32 C to allow full growth of the exconjugants.

Chromosome mobilization. The donors and recipients were grown overnight, in L broth at 32 C on a shaker, to a cell concentration of 2×10^9 to 3×10^9 bacteria per ml. Equal volumes of the donors, undiluted or diluted in L broth, and recipients were then mixed and kept without shaking for 15 min. Samples of 0.1 ml were then plated on minimal or supplemented minimal plates to score for recombinants.

Test for donorability (presence of factor K plasmid). Cells to be tested for the presence of factor K were grown overnight in L broth and patched on an L agar plate. About 9 or 10 patches could be made on one plate. The patches were then grown for 18 h and replica-plated on to minimal or supplemented minimal plates seeded with 0.1 ml of an overnight-grown auxotrophic recipient culture. These plates were then incubated for 3 days at 32 C.

RESULTS

Transfer of OCT and chromosomal markers in P. putida. We have shown previously that the genes specifying enzymes responsible for the degradation of *n*-octane in *P. oleovorans* occur on a plasmid and can be

TABLE 1. *List of bacterial strains*[a]

Strain	Work culture no.	Phenotype	Plasmids	Derivation	Reference
P. oleovorans	PpG6	OCT^+	OCT,K	WT	5
P. oleovorans	AC 1	OCT^+,Met^-	OCT,K	NG	4
P. putida PpG1	AC 2	Trp^-		NG	
P. putida PpG1	AC 4	OCT^+,Trp^-	OCT,K	Conj., AC 1 → AC 2	4
P. putida PpG1	AC 9	Met^-		NG	
P. putida PpG1	AC 12	Ilv^-,Str^r		NG, Spon.	
P. putida PpG1	AC 13	His^-,Str^r		NG, Spon.	
P. putida PpG1	AC 20	Ilv^-,Trp^-		NG	
P. putida PpG1	AC 22	Ilv^-,Leu^-		NG	
P. putida PpG1	AC 32	Met^-,Cam^+	CAM	Conj. PpG1 → AC 9	4
P. putida PpG1	AC 6	Trp^-,Cam^+,OCT^+	CAM,OCT(K)	Conj. AC 32 → AC 4	
P. putida PpG1	AC 41	Ilv^-,Leu^+	K	Conj. AC 4 → AC 22	
P. putida PpG1	AC 38	Trp^-,Ilv^+	K	Conj. AC 75 → AC 20	
P. putida PpG1	AC 75	Met^-,OCT^+	OCT,K	Conj. AC 4 → AC 9	
P. putida PpG1	AC 77	Met^-,OCT^+	OCT	Mit C	
P. putida PpG1	AC 99	Met^-,CAM^+,SAL^+	CAM,SAL	Conj. R1 → AC 32	3
P. putida PpG1	AC 61	Trp^-,CAM^+	CAM	Conj. PpG1 → AC 2	10
P. putida PpG1	AC 60	Trp^-,CAM^+,SAL^+	CAM,SAL	Conj. R1 → AC 61	3
P. putida PpG1	AC 59	Trp^-,CAM-OCT^+	CAM-OCT	Conj. PAO → AC 2	4

[a] Abbreviations used: NG, *N*-methyl-*N*'nitro-*N*-nitrosoguanidine; Spon, spontaneous; WT, wild type; Conj., conjugation; Mit C, mitomycin C.

transferred from *P. oleovorans* to *P. putida* strain PpG1 at a low frequency (4). This can be seen more clearly from the results in Table 2. The OCT^+CAM^- PpG1 exconjugants retained the OCT stably and could transfer it to other PpG1 mutants at a high frequency. An interesting observation in this regard is the prototrophic nature of OCT^+ exconjugants in *P. putida* PpG1. Thus, when OCT is transferred from *P. oleovorans* to different auxotrophic mutants of *P. putida* strain PpG1, most of such OCT^+ exconjugants still retain their auxotrophy. In contrast, when OCT is transferred from *P. putida* strain PpG1 to other PpG1 auxotrophic mutants, about 80 to 90% of such auxotrophic mutants that receive OCT become prototrophic (Table 2). Exconjugants which have been cured of the OCT plasmid remain prototrophic, suggesting that transfers of OCT and chromosomal markers are separate, independent events. The prototrophic OCT^+ exconjugants are therefore chromosomal recombinants that acquire the OCT plasmid by secondary infection.

Incompatibility of CAM and OCT in P. putida. In view of the inability of *P. oleovorans* cells to harbor both CAM and OCT stably (4, 5), we decided to re-examine the segregation of the plasmids in *P. putida* to determine whether such incompatibility could be a function of cellular environment. It is clear (Table 3) that CAM and OCT are incompatible in *P. putida* cells too. Thus, introduction of OCT in culture AC 32 harboring CAM leads to the segregation of CAM, whereas introduction of CAM to AC 4 harboring OCT leads to the segregation of OCT. About 1 to 2% of the exconjugants retain both CAM and OCT for several generations, but continued subculturing on a nonselective medium like L agar plates gives mostly OCT^+CAM^- variants. Selection on camphor

TABLE 2. *Transmissibility of the OCT plasmid*

Donor	Recipient	Selected marker	Transfer frequency	Exconjugant phenotype
AC 1 (OCT^+Met^-)	AC 2 (Trp^-)	OCT^+	10^{-9}	OCT^+,Trp^-
	AC 13 (His^-)	OCT^+	2×10^{-9}	OCT^+,His^-
AC 4 (OCT^+Trp^-)	AC 9 (Met^-)	OCT^+	10^{-5}	OCT^+,Met^+ (80%) OCT^+,Met^- (20%)
	AC 12 (Ilv^-)	OCT^+	5×10^{-6}	OCT^+,Ilv^+ (95%) OCT^+,Ilv^- (5%)
AC 75 (OCT^+Met^-)	AC 2 (Trp^-)	OCT^+	5×10^{-5}	OCT^+,Trp^+ (90%) OCT^+,Trp^- (10%)

TABLE 3. *Incompatibility of CAM and OCT in P. putida*

Donor	Recipient	Plasmid transferred	Resident plasmid	Transfer frequency	Exconjugant phenotype
AC 4 (OCT^+Trp^-)	AC 32 (CAM^+Met^-)	OCT	CAM	3×10^{-7}	$Met^+OCT^+CAM^-$ (93%) $Met^-OCT^+CAM^-$ (6%) $Met^-OCT^+CAM^+$ (1%)
AC 32 (CAM^+Met^-)	AC 4 (OCT^+Trp^-)	CAM	OCT	10^{-7}	$Trp^-CAM^+OCT^-$
AC 99 ($CAM^+SAL^+Met^-$)	AC 4 (OCT^+Trp^-)	SAL	OCT	10^{-4}	$Trp^-OCT^+SAL^+$
AC 99 ($CAM^+SAL^+Met^-$)	AC 4 (OCT^+Trp^-)	CAM	OCT	2×10^{-6}	$Trp^-CAM^+OCT^-$ (98%) $Trp^-CAM^+OCT^+$ (2%)
AC 4 (OCT^+Trp^-)	AC 99 ($CAM^+SAL^+Met^-$)	OCT	CAM,SAL	10^{-7}	$Met^+OCT^+CAM^-SAL^-$ (95%) $Met^+OCT^+CAM^-SAL^+$ (5%)

plates, however, produces slow-growing CAM^+ cells that no longer retain OCT. One interesting property of those CAM^+OCT^+ unstable exconjugants is that, unlike those in *P. oleovorans*, most of the CAM^+OCT^+ *P. putida* exconjugants can no longer transfer either CAM or OCT, except at very low frequency. Segregation of one of the plasmids restores transfer function for the other plasmid, suggesting that there is a mutual repression of transfer function for both of these plasmids as a result of their joint coexistence. Also, OCT seems to be compatible with SAL when SAL is the only plasmid present in the recipient cells, but introduction of OCT to recipients harboring both CAM and SAL leads to the enhanced segregation of SAL along with CAM.

The ability of OCT^+ exconjugants of *P. putida* to donate chromosomal markers to other *P. putida* recipients has been analyzed further because of their value in the fine-structure mapping of the chromosome. The OCT^+Trp^- *P. putida* exconjugant (AC 4) that recieved the OCT plasmid from *P. oleovorans* acts as a potent donor and can donate a variety of chromosomal markers at a frequency of 10^{-2} to 10^{-3} per donor cell (Table 4). Most of the recombinants are stable prototrophs and do not undergo further segregation, either spontaneously or on treatment with a number of curing agents, suggesting that they are presumably true haploid cells. Most of the recombinants are also OCT^-. However, even though the recombinants do not acquire OCT plasmid, a significant proportion (40 to 80%) of them acquire the donorability, so that they in turn can donate chromosomal markers to other recipients (experiments 3 and 4, Table 4).

Curing of the infectious plasmid. The differential acquisition of the donorability of chromosomal markers, but not the OCT plasmid, raises the interesting questions as to whether they are in fact different plasmids and, if this is so, whether one of them could be eliminated from the cell without the loss of the other. It is also pertinent to ask if the plasmid responsible for chromosome mobilization (infectious plasmid) would in fact have any role in the trans-

TABLE 4. *Transfer of chromosomal markers by OCT^+ exconjugants and by recombinants*

Expt	Donor	Recipient	Marker selected	Frequency of recombination
1	AC 4 (OCT^+Trp^-)	AC 9 (Met^-)	Met^+	5×10^{-3}
		AC 12 (Ilv^-)	Ilv^+	2×10^{-2}
		AC 13 (His^-)	His^+	3×10^{-3}
		AC 20 (Ilv^-Trp^-)	Ilv^+	7×10^{-4}
			Trp^+	1×10^{-4}
		AC 22 (Ilv^-Leu^-)	Ilv^+	5×10^{-4}
			Leu^+	1×10^{-4}
2	AC 75 (OCT^+Met^-)	AC 2 (Trp^-)	Trp^+	4×10^{-3}
		AC 12 (Ilv^-)	Ilv^+	1×10^{-2}
3	AC 38 (Ilv^+Trp^-)	AC 13 (His^-)	His^+	1×10^{-4}
4	AC 41 (Ilv^-Leu^+)	AC 2 (Trp^-)	Trp^+	3×10^{-4}
		AC 9 (Met^-)	Met^+	1×10^{-4}

missibility of the OCT plasmid. An attempt was made, therefore, to cure the infectious plasmid responsible for chromosome mobilization, without loss of the OCT plasmid. This was achieved by treating OCT^+Met^- AC 75 cells with mitomycin C and examining a large number of OCT^+-treated cells for their ability to act as donors against one or two auxotrophic mutants as recipients (Table 5). It is clear that mitomycin C can independently cure either of these two plasmids. A few OCT^- segregants appeared to have lost the infectious plasmid as well. All the segregants that have lost the ability to act as genetic donors still retained a functional OCT plasmid. Thus, the OCT plasmid must be different from the infectious plasmid, which is solely responsible for the mobilization of chromosome. We wish to designate this infectious plasmid as factor K.

Mobilization of noninfectious OCT plasmid by factor K. The role of factor K in the mobilization of chromosomal markers at a high frequency is reminiscent of other infectious plasmids like F in *Escherichia coli* or FP in *P. aeruginosa* (7, 8). Since this plasmid was obtained only during transfer of OCT from *P. oleovorans* to *P. putida*, but not during mobilization of other infectious plasmids like CAM or SAL from *P. oleovorans* (to which CAM or SAL was introduced before) or during transfer of other plasmids like SAL from other *Pseudomonas* species to PpG1, it was of interest to find out if factor K would have a role in the transfer of OCT plasmid itself. The results (Table 6) indicate that the mitomycin C-induced segregant, which lost factor K (AC 77), is no longer capable of transferring either chromosomal markers or the OCT plasmid to a variety of recipients. The reintroduction of factor K in these cells restores their ability to transfer both chromosomal genes and the OCT plasmid. The inability of AC 77 lacking factor K to transfer chromosomal markers and the OCT plasmid is not due to its general inability to transfer any plasmid, since it is perfectly capable of transferring CAM or SAL at a high frequency (10^{-2} to 10^{-3} per donor) when these plasmids are introduced into it. Interestingly, factor K appears to be compatible to CAM, in contrast to OCT, since introduction of CAM leads to the enhanced segregation of OCT but not of K from cells harboring both OCT and K. Thus, 9 out of 10 CAM^+OCT^- segregants of AC 75 could transfer chromosomal markers at a high frequency, suggesting the retention of K. Also, AC 59, which harbors the fused CAM-OCT plasmid, where both CAM and OCT plasmids have undergone recombination to become part of the same replicon (4), lack any K activity, suggesting that the fusion involves only the OCT plasmid but not factor K. It therefore seems that OCT is a plasmid aggregate, in contrast to CAM or SAL, which appear to be plasmid co-integrates (6). We have recently characterized another infectious plasmid (MER, responsible for conferring resistance to inorganic mercury ions) that is transferred from *P. oleovorans* to *P. putida* with OCT and factor K and which is incompatible with CAM. The interactions of these plasmids and the role of other infectious plasmids in the transfer of OCT as well as the ability of factor K to initiate transfer of other noninfectious plasmids in *Pseudomonas* will be the subject of out next communication.

DISCUSSION

The dissociation of infectious OCT plasmid into an infectious plasmid (factor K) and a noninfectious OCT plasmid is reminiscent of the drug-resistance factors Δ^+A^+ or Δ^+S^+, which are composed of an infectious plasmid, Δ, and the noninfectious resistance determinants A or S (1, 2). On the other hand, drug-resistance factors are known (Δ-T) in which the resistance genes are part of the infectious plasmid (9).

TABLE 5. *Curing of the infectious plasmid and the OCT plasmid by mitomycin C*

Strain	Mitomycin C concn (μg/ml)	Plasmid cured	Frequency of curing (%)	Phenotype of cured cells[a]
AC 75 (K^+OCT^+)	0	OCT	<1.0	
	2	OCT	1.0	OCT^-,K^+
	4	OCT	1.7	OCT^-K^+(95%)OCT^-K^-(5%)
	6	OCT	2.1	OCT^-K^+(90%)OCT^-K^-(10%)
	0	K	<1.0	
	2	K	0.5	K^-,OCT^+
	4	K	1.0	K^-,OCT^+
	6	K	2.2	K^-,OCT^+

[a] Presence of factor K has been determined by the ability of the cured cells to donate chromosomal markers to suitable auxotrophic recipients.

TABLE 6. *Inability of OCT⁺K⁻ cells to donate either chromosomal markers or OCT*

Donor	Recipient	Selected marker	Frequency of transfer
AC 75 (OCT⁺Met⁻)	AC 2 (Trp⁻)	OCT	2×10^{-6}
		Trp⁺	7×10^{-3}
	AC 12 (Ilv⁻)	OCT	1×10^{-5}
		Ilv⁺	2×10^{-2}
AC 77 (OCT⁺K⁻)	AC 2 (Trp⁻)	OCT	$<1 \times 10^{-8}$
		Trp⁺	$<1 \times 10^{-8}$
	AC 12 (Ilv⁻)	OCT	$<1 \times 10^{-8}$
		Ilv⁺	$<1 \times 10^{-8}$
K⁺AC 77[a]	AC 2 (Trp⁻)	OCT	7×10^{-5}
		Trp⁺	4×10^{-4}

[a] Factor K has been introduced into K⁻AC 77 by using K⁺MDL⁺Trp⁻AC 117 as a donor and selecting exconjugants on mandelate plates containing methionine. A Met⁻MDL⁺OCT⁺K⁺ exconjugant was purified from which a MDL plasmid was eliminated by mitomycin C treatment.

Clowes has coined the term plasmid aggregate for the former, whereas the latter are referred to as plasmid co-integrates (6). Thus, OCT appears to be a plasmid aggregate, whereas CAM and SAL seem to behave like plasmid co-integrates, although rigorous proof in this respect is lacking because of our inability to isolate these degradative plasmids physically separated from the chromosome (3). Transfer of OCT by factor K is also analogous to the transfer to noninfectious plasmids like Col E1 and Col E2 by Col I (12) and F (6) and other noninfectious drug-resistance, colicinogeny, or α-hemolysin plasmids by the infectious sex factor F (11). Introduction of CAM or SAL into cells harboring OCT, but not factor K, does not lead to transfer of OCT at any appreciable frequency, suggesting that the transfer factors in these plasmids cannot initiate OCT transfer. We have characterized another infectious plasmid (MER, responsible for conferring resistance to a high concentration of mercury salts; see above) which is transferred along with OCT, can be cured without loss of OCT or factor K, appears to be incompatible with CAM, and, thus, might be a component of the OCT plasmid aggregate. This plasmid, although infectious, does not enhance OCT transfer in the absence of factor K, suggesting that the transfer genes must specify protein components which are highly specific for each plasmid. As yet, we have no evidence of the occurrence of surface appendages specific for these plasmids. Information about the interactions and physical structure of the component plasmids of the OCT plasmid aggregate would be very useful in understanding the mode of replication and the compatibility characteristics of these plasmids in *Pseudomonas*.

The occurrence of factor K as the transfer plasmid in *P. putida* has been extremely useful not only in dissecting the genetic anatomy of the OCT plasmid aggregate, but also in providing us with a technique for overall mapping of the *P. putida* chromosome. The high frequency with which chromosomal markers are transferred (10^{-2} to 10^{-3} per donor) because of the presence of factor K truly confers on it the role of a sex factor plasmid. In this respect, factor K resembles the infectious sex factor plasmids FP 2 and FP 39 of *P. aeruginosa* (8), which have been successfully used to construct a detailed genetic linkage map of this species based on the time of entry of individual genetic markers during an interrupted mating. However, although infectious, the sex factors FP 2 and FP 39 have not been reported to lead to the transfer of any noninfectious plasmid in *P. aeruginosa*, and several attempts to transfer them to *P. putida* or other species of *Pseudomonas* have failed. A direct comparison of the similarities of FP 2 or FP 39 and factor K, their compatibility, or their interaction is therefore not possible at this time. Attempts are presently being made in our laboratory to devise an interrupted mating technique for determining the time of entry of a number of chromosomal genes. Another very useful role of factor K is the transfer of noninfectious plasmids in *P. putida*. We have often encountered genetic determinants that do not seem to map on the chromosome and seem to undergo loss of expression or existence on introduction of some plasmids, but rigorous proof of their extrachromosomal nature, viz., transmissibility or curing by usual curing agents, is lacking. Introduction of factor K into such cells has helped us, at least in one instance, to demonstrate the plasmid nature of such a genetic determinant (A. M. Chakrabarty, unpublished observation). It is likely that the introduction of factor K into cells harboring noninfectious plasmids will lead to more complete characterization and the study of a number of such extrachromosomal elements.

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