MOLECULAR CHARACTERIZATION OF HYDROCARBON DEGRADATIVE PLASMIDS IN *PSEUDOMONAS PUTIDA*

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SUMMARY. Plasmids detected in soil *Pseudomonas* species allow growth on complex organic compounds including CAM (camphor), SAL (salicylate), and OCT (octane), plus a fertility factor K or P. The double stranded, covalently closed circular DNA was isolated from Oct^+, Cam^+, Sal^+, and K^+ strains, purified on cesium chloride-ethidium bromide gradients and converted into open circular form for electron microscopy. Their contour lengths in μm were: OCT, 14; fertility factor, 31; CAM, 78; SAL, 18, 26, 31.3, and 48.8. The Oct^+K^+ (Oct^+P^+) strains always contained two autonomous circular duplex DNA molecules, 14 and 31 μm.

Genus *Pseudomonas* strains are known to use a wide range of complex organic compounds as a source of carbon and energy (1). Nutritional versatility must have a genetic basis; recent findings indicate that plasmids may provide host strains with such extra metabolic activities (2). Genes coding for the oxidation of n-octane to octanoic acid (or beyond) seem to be borne on an extrachromosomal genetic element, /OCT (2). Oct^+ donors of *P. putida* transfer most chromosomal markers without the simultaneous transfer of the OCT plasmid phenotype. Similar to F factor in *E. coli* K12 the fertility factor P or K has been postulated to initiate chromosomal mobilization (3, M. Hermann, personal communication) and at a lower frequency transfer the Oct^+ genes. Additional examples of extrachromosomal elements are the camphor degradative pathway of 15-20 inducible enzymes which appear to be transferred on a self-transmissible element /CAM (4) and in another strain of *Pseudomonas putida* salicylate dissimilation also clustered on an extrachromosomal element /SAL (5). SAL, like CAM and OCT, is curable by mitomycin C and transmissible to a variety of *Pseudomonas* species by conjugation. Enzymes of the aromatic meta-cleavage pathway are induced by salicylate, not by catechol, and correlate with the presence of /SAL plasmid (5).

Until recently, evidence for the extrachromosomal nature of all these known hydrocarbon metabolizing plasmids of *P. putida* was purely genetic. Our recently published method (6) for the isolation of *Pseudomonas*-plasmids has permitted their molecular characterization together with that of the transfer factor K or P. This report confirms the existence of OCT, CAM,

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SAL and the transfer factor as covalently closed circular (ccc) molecules of duplex DNA. In addition, the contour lengths of these molecules are measured by electron microscopy.

MATERIALS AND METHODS. All bacterial and bacteriophage strains used, and their phenotypes, genotypes and sources, are mentioned in Table 1. The minimal medium routinely employed for mating experiments was PAS (7); and the complete medium, L-broth (6), was used for the conjugation and transduction experiments. Stocks of the phage pf16 were prepared by the agar overlay technique using a low multiplicity of infection (10^{-2}). For mating experiments the donor and recipient cells were grown on L-broth, mixed in a 1:1 ratio, and samples were withdrawn for plating on appropriate supplemented media. The parental donor strain was counterselected against its auxotrophic markers. A replica mating (8) was used to test a large number of individual donor strains.

Plasmids were cured with mitomycin C following the method of Rheinwald et al. (4). The preparation of plasmid DNA from *P. putida* cells carrying hydrocarbon metabolic plasmids or drug resistant factors has recently been published (6). The strains were grown from single colony isolates in enriched minimal media as described previously (6); and the plasmid DNA was isolated and purified by two sequential buoyant density centrifugation runs in CsCl in the presence of ethidium bromide. Before determining the contour lengths, the supercoiled DNA samples were treated at room temperature with x-ray doses ranging from 50 to 1000 rads to give the open circular forms (9) and then mounted by the aqueous technique for electron microscopy (10) to determine homogeneity, the approximate DNA concentration and the length of the plasmid molecules. Molecular lengths were determined relative to double stranded circular ColE1 DNA, whose molecular length is 2.1 μm and molecular weight 4.2×10^6 daltons (10).

RESULTS. The coexistence of the nontransmissible OCT plasmid and a fertility factor (K or P) in Oct^+K^+, or Oct^+P^+, strains has been confirmed by the genetic and physical experiments shown in Tables 2 to 4.

Most of the recombinants selected for Oct^+ or His^+ from matings of Oct^+K^+ donors with suitable recipients, Table 2, still carried the fertility factor, but all of 50 His^+ selected recombinants were Oct^-. To determine whether the fertility factor responsible for chromosomal mobilization plays a role in the transmissibility of the OCT plasmid, or if K and OCT exist as a fused plasmid (11), 100 Oct^+ recombinants, purified by single colony isolation, were treated with an appropriate concentration of mitomycin C for curing. The segregants, Oct^+K^- (SP8), Oct^-K^+ (SP5 and SP6) and Oct^-K^- (SP7), thus obtained, were checked for donor ability. These data, Table 2, indicate that the fertility factor cotransfers the OCT plasmid which by itself is nontransmissible. The data shown in Tables 2 and 3 are in accord with the results of previous workers (3 to 5), except that SAL shows a low frequency of transfer of chromosomal markers. The SAL and CAM plasmids were transduced at a low frequency by phage pf16 (data not shown), and the Sal^+, but not the Cam^+, transductants retain the conjugal transfer capability with suitable recipients.

Table 1

List of bacterial strains

Strain designation	Phenotype	Plasmids	References or sources
AC34	Ade^-	Absent	(6), PpG1361
AC36	$Ade^- Sal^+$	SAL	(6), AC34 + Sal conjugation
AC34-1	$Ade^- Sal^-$	Absent	(6), AC36 + MC* curing
SP1	$Ade^- Cam^+$	CAM	PpG273 x AC34 selecting for Cam^+
SP2	$Ade^+ Cam^+$	CAM	Ade^+ recombinant in the above mating
PpG273	$Trp^- Cam^+$	CAM	(4), G. Jacoby
PpG1400	$Trp^- Rec^-$ (Cam^-)	Absent	†, M. Hermann
SP3 rec104	$Trp^- Cam^+$	CAM	SP1 x PpG1400 selecting for Cam^+
PpG1404	$Trp^- Rec^-$ (Cam^-) (Oct^+)	OCT, P	†, M. Hermann
PpG972	$Trp^- Oct^+$	OCT, P	(16), G. Jacoby
SP5	$Trp^- Oct^-$	P	PpG972 + MC* curing
AC10	Oct^+, Met^-	OCT, K	(17), A. Chakrabarty
SP6	$Oct^- Met^-$	K	AC10 + MC* curing
SP7	$Oct^- Met^-$	Absent	AC10 + MC* curing
SP8	$Oct^+ Met^-$	OCT	AC10 + MC* curing
AC536	$Ben^- His^-$	Absent	A. Chakrabarty
Phage pf16			(18), A. Chakrabarty

* MC = Mitomycin C

† Hermann, M., Garg, G. K. and Gunsalus, I. C. "Fertility Factors for High Frequency Chromosome Transfer in *Pseudomonas putida*. Prepared for publication in J. Bacteriol.

Five hundred ml cultures of the Oct^+ strains, PpG972, AC10 and PpG1404 in mid log phase at a titer of 5×10^8/ml, were extracted by our normal procedure (6) and the DNA purified by centrifugation to equilibrium in CsCl-ethidium bromide density gradients. In each case a "satellite" component of higher density than the bulk (chromosomal and open circular) DNA representing

Table 2
Conjugal transfers

Donor	Recipient	Selection*	Recombinants or exconjugants/donor
PpG972 (Oct^+ P^+)	AC536	Oct^+ His^+	1×10^{-5} 1×10^{-3}
SP5 (Oct^- P^+)	AC536	Oct^+ His^+	$0 (< 10^{-8})$ 2×10^{-3}
AC10 (Oct^+ K^+)	AC536	Oct^+ His^+	2×10^{-5} 1×10^{-3}
SP6 (Oct^- K^+)	AC536	Oct^+ His^+	$0 (< 10^{-8})$ 2×10^{-3}
SP8 (Oct^+ K^-)	AC536	Oct^+ His^+	$< 10^{-8}$ $< 10^{-8}$
PpG273 (Cam^+)	AC536	$D\text{-}Cam^+$ His^+	2×10^{-5} 5×10^{-6}
AC36 (Sal^+)	AC536	Sal^+ His^+	1×10^{-3} 1×10^{-8}
AC34-1 (Sal^-)	AC536	Sal^+ His^+	$< 10^{-8}$ $< 10^{-8}$

*Selection of exconjugants and recombinants occurred on normal medium which included appropriate amino acids and one relevant carbon source.

plasmid DNA was examined with the electron microscope. In each case about 99% was double stranded, covalently closed circular (ccc) DNA. These samples were subjected to x-ray treatment, and the open circular DNA, thus formed, was measured for contour length with the electron microscope. Two sizes of molecules were distinguished respectively with contour lengths 14 and 31 μm as shown in Table 4. To determine which, if either, is responsible for the oxidation of n-octane, we isolated the plasmid DNA from the partially cured and completely cured strains (SP5, 6, and 7). Electron microscopy confirmed the presence of the 31 μm molecule in Oct^-K^+ and Oct^-P^+ strains; in the completely cured strains, Oct^-K^-, the dense bands were not observed in CsCl-ethidium bromide equilibrium gradients (see Table 4). Thus the molecule of contour length 14 μm is apparently associated with the Oct^+ phenotype.

Table 3

Curing of the OCT, CAM and SAL plasmids with Mitomycin C

Strains	Plasmid	Mitomycin C concentration (μg/ml)	Frequency of curing	Phenotype of cured strains
AC10	OCT	0	0	
		5	1.7	
		9	2.4	Oct^- Met^-
SP6 or SP5	K (or P)	0	0	
		5	1.5	
		9	2.7	Met^- *
SP2	CAM	0	0	
		5	45	
		9	90	Cam^-
AC36	SAL	0	0	
		5	0.9	
		9	2.0	Sal^- Ade^-

*Presence of fertility factor has been determined by the ability of the cured cells to donate chromosomal markers.

CAM plasmid was isolated from strains PpG273, SP1, and SP3 recl04 and characterized by the presence of one large circular DNA of contour length 78 μm. The Cam^- isogenic strain did not reveal a band (plasmid DNA). One Cam^+ strain, SP1, did show a predominance of 31 μm and 49.3 μm length DNA molecules and, interestingly, a minor percentage of a larger molecule (78 μm), Table 4. The cells of the Sal^+ phenotype, AC36, harvested from 500 ml cultures, titer = 5×10^8/ml, gave a much higher yield of plasmid DNA (6). The majority of the molecules were of contour length 26.5 μm, whereas about 5% of the total number consisted of molecules of three different lengths, i.e., 48.8, 31.4, and 18 μm as shown in Table 4. The SAL cured strain, AC34-1, Sal^-, Ade^-, derived from AC36, did not exhibit the more dense ccc-DNA band in ethidium bromide equilibrium density banding.

DISCUSSION. The hydrocarbon metabolic plasmids represent a new type of extrachromosomal element which may contribute significantly to the nutritional versatility in soil pseudomonads. Most of these plasmids are conjugative among a rather large number of *Pseudomonas* species. Currently

Table 4
Plasmids present in different strains of P. putida

Source of plasmid DNA	Degradative pathway	Size of plasmid molecules (μm)			
PpG972	n-octane	31.0 (15)	14.0 (15)		
PpG1404	n-octane	31.0 (12)	14.0 (16)		
AC10	n-octane	31.0 (12)	14.5 (11)		
SP5 (Oct^- P^+)	-	31.0 (25)	absent		
SP6 (Oct^- K^+)	- -	31.0 (24)	absent		
SP 7 (Oct^- K^-)	-	absent	absent		
PpG273	Camphor	78.0 (25)	-		
SP 3 rec104	Camphor	78.0 (25)	-		
SP 3 rec104	Camphor	79.5 (12)	-		
SP 1	Camphor	78.0 (6)	49.0 (10)	31.0 (10)	
AC36	Salicylate	18.0 (20)	48.8 (10)	31.0 (14)	26.5 (40)

The numbers in parenthesis indicate the number of molecules measured.

it is unclear whether or not different degradative plasmids contain common DNA sequences for conjugative transfer or replication functions. The K (or P) fertility factor can be compared with the *E. coli* sex factor, F, in the mobilization of host chromosomal markers at a high frequency, 10^{-2} to 10^{-3} per donor. The K (or P) fertility factor possesses an additional and important capacity, that is, the ability to transfer the non-transmissible OCT-plasmid.

The Cam^+ phenotype appears to be associated with a single large plasmid, 78 μm, with the exception of strain SP1 which contains circular duplex DNA of three contour lengths: 78, 49, and 31 μm. Thus, one may conclude that

the CAM plasmid, 78 μm, is unstable in this genetic background and dissociates possibly into a CAM determinant segment of 49 μm and a fertility factor of 31 μm, possibly K. The latter would be analogous to the resistance factor ΔA composed of a transfer factor, Δ, and the nontransmissible resistance determinants, A (12). Sal^+ cells appear to contain a variety of plasmid DNA in strain AC36 ranging in sizes, 48.8, 31.4, 26.5, and 18.0 μm, as shown in Table 4. Again the numbers may suggest that the 48.8 μm fragment could be formed by the reversible association of 31.4 and 18.0 μm fragments. At this stage an approximation for the 26.4 component is not offered. Since the phage pf16 genome is about 100×10^6 daltons (Niblack and Gunsalus, personal communication; Palchaudhuri, unpublished data), it is possible that any of the fragments appreciably below this in size, perhaps including the 48.8 (96 million dalton) SAL fragment, may be transduced by this phage.

Compatability is widely used in characterizing bacterial plasmids. Thus it is important to know if the transfer systems for the metabolic plasmids are aggregates (13) among the plasmids studied here. Suggestion arises, as shown in Table 4, that each, in some strain backgrounds at least, may exist as plasmid aggregates and thus decisions as to incompatability among plasmid determinants which are independent of the transfer factors may lead to confusion. The ccc-DNA molecules isolated from XYL 14 and NAH 15 containing strains appear to be heterogeneous though we infer that in each case a molecule of contour length about 31 μm, possibly a fertility factor, is present. More complete results will be reported elsewhere.

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