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Genetic Regulation of Octane Dissimilation Plasmid in *Pseudomonas*

(incompatibility)

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ABSTRACT **The enzymes responsible for the oxidation of *n*-octane to octanoic acid or beyond in *Pseudomonas oleovorans* are octane inducible and are coded by genes borne on a transmissible extrachromosomal element. The octane to octanoate enzymes induced by octane are repressed by octanol. The chromosome also carries genes coding octanol oxidation enzymes that, in contrast, are induced by octanol, not by octane. The octane plasmid has been transferred from *P. oleovorans* to several other fluorescent *Pseudomonas* species. In exconjugants, the presence of both octane and camphor plasmids enhances their segregation rate.**

The nutritional versatility of *Pseudomonas* species to proliferate on diverse organic compounds not normally used by other bacteria has attracted much attention and analysis (1–5). An extreme example is a *Pseudomonas multivorans* strain that utilized 108 of 146 different complex organic compounds tested—an average among fluorescent species is about 80, or nearly 60% (5). Biochemists and enzymologists have exploited this versatility to study the regulation of a number of complex catabolic pathways within a single strain (4). Clearly these cells are endowed with the genetic information to specify the many sets of enzymes required for the catabolic processes. Nevertheless, the *Pseudomonas* genome is essentially of the same size as that found in the enteric bacteria (6, 7). We undertook an analysis of the disposition of the genes coding catabolic pathway enzymes in several *Pseudomonas* species. Many of these genes are associated with extrachromosomal elements rather than with the chromosome. Thus, the dissimilation of camphor is specified by a transmissible plasmid in *Pseudomonas putida* strain PpG1 (8–10), as are salicylate dissimilation in *P. putida* strain R1 (11) and naphthalene in strain PpG7 (12). Since salicylate is an intermediate in naphthalene oxidation (ref. 11, p. 20), the relationship between these two elements presents an interesting case for analysis. We were unable to "cure" naphthalene use and to demonstrate an extrachromosomal array in one of two strains tested. Similarly, the genes specifying mandelate to benzoate oxidation in PpG3 (Stanier's strain A3.12) (4, 13) have not been shown to segregate and appear to be chromosomal. This gene cluster, however, on transfer to the PgG2 strain is carried as an autonomous extrachromosomal element (10, 13).

In this report, we present evidence that the *P. oleovorans*‡ strain of Coon and associates (17) carries on a "curable" extrachromosomal element the genes coding for the inducible enzymes responsible for hydrocarbon oxygenation, e.g., *n*-octane, and octanol. This plasmid can be transferred to several other fluorescent pseudomonads, though at low frequency. The segregation rate is increased by the presence of other extrachromosomal elements.

TABLE 1. *Wild-type and derived strains used**

P-G no.	Genotype	Phenotype	Parent	Treatment (ref.)
1	wt	Cam^+	–	Enrich (10)
6	wt	Oct^+	–	Enrich (17)
8	wt	Phl^+	–	Enrich (14–16)
273	*trpB615*/CAM	Trp^-Cam^+	1	PC(10)
572	/CAM^d	Cam^-	1	PC(10)
968	/OCT^d	Oct^-	6	MC
800	*trp*-901/OCT	Trp^-Oct^+	6	NG
801	/CAM,OCT^d	$Cam^+Oct^-Ocl^-$	273 × 6	C
802	/CAM,OCT	$Cam^+Oct^+Ocl^-$	800 × 801	C
a10	wt	($Cam^-Oct^-Ocl^+$)	–	–(10)
a12	wt	($Cam^-Oct^-Ocl^+$)	–	–(10)

* For abbreviations and symbols, see footnote and ref. 10.

MATERIALS AND METHODS

Bacterial Strains. The phenol-oxidizing strain U of *P. putida* was isolated from the Boneyard Creek in Urbana by Dr. Stanley Dagley (14, 15)—Stanier collection *P. putida* biotype A, number 144, ATCC 17514, NTCC 10105 (see ref. 16). This culture was kindly supplied by Dr. George Hegeman of the University of California, Berkeley; we assigned the number PpG8. *P. oleovorans*, strain PpG6, was isolated by enrichment technique on hexane as the sole source of carbon (17). These cultures and derivatives are listed with their properties in Table 1.

Media and Growth Condition. P. oleovorans, and other strains that acquired the ability to grow on *n*-octane as the

Abbreviations: Phenotypes, Genotypes, and Plasmids, respectively, of cells that can degrade the following compounds are: Camphor—Cam, *cam*, CAM; Octane—Oct, *oct*, OCT; Octanol (1-octanol)—Ocl, *ocl;* and Phenol—Phl, *phl.*

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‡ The Coon strain of *P. oleovorans* has been shown to conform to the properties of *P. putida* biotype A by Stanier *et al.* (5) under the number 266 (ATCC 17633, our collection PpG6).

TABLE 2. *Cam+ and Oct+ plasmid transfer by conjugation*

Donor × rec.	Phenotype		Frequency per donor × 10^n
	Recipient	Exconjugant	(n =)
273* × 572	Cam^-Oct^-	Cam^+Oct^-	5
273 × a12	Cam^-Oct^-	Cam^+Oct^-	6
273 × 6	$Cam^-Oct^+Ocl^+$	$Cam^+Oct^-Ocl^{-/+}$§	9
273 × 968	$Cam^-Oct^-Ocl^+$	$Cam^+Oct^-Ocl^{-/+}$§	7, 8
800† × 8	Oct^-Phl^+	Oct^+	7
800 × 801	$Cam^+Oct^-Ocl^-$	$Cam^+Oct^+Ocl^{-/+}$¶	8
800 × 968	Oct^-Ocl^+	Oct^+Ocl^+	9
800 × 1	Cam^+Oct^-	Oct^+	9
800 × a10	Oct^-	—	>9

* PpG 273 = Trp^-Cam^+; † PpG 800 = Trp^-Oct^+; § $Ocl^{-/+}$ = exconjugant majority type Ocl^-, minority type Ocl^+; ¶ Exconjugant $Ocl^{-/+}$ = 90/10 ratio.

sole source of carbon by genetic exchange, were cultured on a synthetic minimal medium (PAS) containing per liter: respectively, 25 and 12.5 ml of 1 M K_2HPO_4 and KH_2PO_4, 40 ml of 1 M NH_4Cl, and 10 ml of a salt solution. The salt solution contained per liter: 19.5 g of $MgSO_4$, 5 g of $MnSO_4 \cdot H_2O$, 5 g of $FeSO_4 \cdot 7H_2O$, 0.3 g of $CaCl_2 \cdot 2H_2O$, and 1 g of ascorbic acid. The salt solution was sterilized by filtration through 0.22-μm Millipore filters and was added to the autoclaved and cooled mineral salts medium along with other supplements. For growth on minimal octane plates, the mineral medium contained 1.5% agar. Plates were poured and allowed to solidify; a 1-cm strip of agar was removed from one side of the plate and 1–2 ml of octane was added. Octane-positive cells grew under these conditions within 24 hr by uptake of octane vapor.

Curing with Mitomycin C. Mitomycin C has been shown to enhance the fraction of pseudomonad cells that segregate plasmid borne genes (10–12). The effective concentration is a function of strain and of the particular plasmid. The procedure used previously in our experiments has been used here (10–12). Briefly, 2 ml of L-broth in 10-mm test tubes was supplemented with mitomycin C at 5-μg increments to 30 μg/ml. After inoculation with about 10^5 cells of octane-positive *P. oleovorans*, the tubes were incubated at 30° with shaking for 2–4 days. Usually after a lag period of 12–36 hr, appreciable growth appeared. The cultures were diluted and plated on L-agar, then replicated to glucose- and octane-minimal plates to allow scoring and calculation of the proportion of octane-negative segregants.

Conjugational Transfer of Plasmids. The camphor plasmid can be transferred to *P. oleovorans* and to other *Pseudomonas* strains from auxotrophic mutants, e.g., Trp^- to permit counterselection (10). The cells are grown at 30°; the donor in L-broth as a stationary culture for 16 hr or more and the recipients on a shaker for 5–8 hr. Equal volumes of the donor and recipient are mixed and allowed to stand quietly for 30 min; aliquots are plated on selective media at appropriate donor dilutions to yield 100–500 clones per plate. The age of the donor culture is critical for octane-plasmid transfer from strain 6. Transfer was not observed with cultures younger than 18 hr; the optimum was at least 22 hr.

RESULTS AND DISCUSSION

Octane segregation and curing

The intermediates and enzymes for conversion of *n*-octane to octanoate via 1-octanol and octanaldehyde by strain 6 were identified by Coon *et al.* (17). The octane–octanol conversion requires a monoxygenase system composed of three proteins: a flavoprotein, DPNH:rubredoxin reductase; rubredoxin, an iron–sulfur protein without labile sulfide; and the hydroxylase termed ω-hydroxylase (18–20). The octanol to octanoate conversion proceeds by two DPN-specific dehydrogenases—one for alcohol and the second for aldehyde. All of these enzymes are induced by the hydrocarbon octane, whereas octanol and octanoate will not induce the octane oxygenase and, in fact, these products appear to decrease the oxygenase level of late-stationary-phase cultures (18, 19). Octanoate metabolism has not been studied in detail, but is believed to occur by β-oxidation to acetate, then via the glyoxalate acid cycle (16, 21).

The octane phenotype in *P. oleovorans* was observed to be unstable when we introduced the camphor genes by conjugation. As shown in Table 2, the transfer of the camphor plasmid, CAM, was accomplished with the donor 273, a Trp^- auxotroph. The frequency with the recipient 6 was very low, about 10^{-9}, and the exconjugants were found to be octane-negative. The majority, about 93%, were also octanol-negative; all grew on octanoate. The transfer of the camphor plasmid to an octane-negative derivative of strain 6, number 968, prepared by mitomycin C treatment, occurred at 10- to 100-times higher frequency per donor, with similar distribution of the octanol − and + phenotypes. For comparison, *P. aeruginosa*, strain PaG12 and *P. putida*, strain 572, a camphor-cured, CAM-deleted strain—prepared from PpG1—were tested as recipients. The conjugation frequency for CAM donors were 10^{-5} and 10^{-6}, as shown in Table 2—these compare reasonably with the earlier experiments (10). To determine whether the introduction of the camphor plasmid was accomplished by loss or lack of expression of the octane genes, several $Cam^+Oct^-Ocl^-$ exconjugants were purified by single-colony isolation and the camphor plasmid was subsequently removed from the exconjugants either by spontaneous segregation or by treatment with mitomycin C (Table 3). When 10^8 cells of the $Cam^+Oct^-Ocl^-$ exconjugants were spread on minimal plates with octanol as the sole source of carbon, 500–1000 colonies appeared after 2 days of incubation. In contrast, octane-positive cells were never observed, even when 10^9 cells were used. The Ocl^+ revertant colonies were still octane-negative and were found to be cured of the camphor plasmid. It thus seems that introduction of the camphor plasmid leads to the segregation of the octane genes and an inhibition of expression of the octanol gene(s). When the camphor plasmid is removed, such exconjugants regain the Ocl^+ phenotype expression, but not octane. If the octane hydroxylase and octanol dehydrogenase genes were coded by plasmid, they might also be removed from *P.*

TABLE 3. *Camphor plasmid inhibits octanol phenotype*

Strain	Treatment	Phenotype
801	None	$Cam^+Oct^-Ocl^-$
	Mitomycin C, 20 μg/ml	$Cam^-Oct^-Ocl^+$
	Selection on octanol	$Cam^-Oct^-Ocl^+$

oleovorans cells by treatment with some curing agents, as shown previously for camphor (10), salicylate (11), and naphthalene (12). Mitomycin C greatly enhanced the octane segregation rate, and revertants were not obtained from these progeny. The segregants grew well, however, on octanol, suggesting the presence of octanol dehydrogenase and later pathway genes on the chromosome. In *P. oleovorans*, mitomycin C increased the frequency of octane-"cured" strains—the percentages were, at concentrations of mitomycin C in μg/ml; 5, <1%; 10, 1%; 20, 3%; 27, 7%; 30, no growth. These values are low compared to camphor (10), but are similar to naphthalene (12).

Transmissibility of the octane plasmid

The accumulating evidence for the transmissibility of plasmids, not only in *Escherichia coli* for F, colicins, and resistance transfer factor (RTF) (22), but also among the pseudomonads, as shown by our earlier experiments on camphor (10), salicylate (11), and naphthalene (12) oxidation pathway genes, led us to examine *P. oleovorans* for possible transfer of the octane phenotype. The incompatibility of Oct$^+$ and Cam$^+$ when the plasmid for the latter is transferred to *P. oleovorans* and the increased rate of formation of Oct$^-$ strains on mitomycin C treatment are presumptive evidence of an extrachromosomal array for these genes. Table 2 shows the transfer of the octane character from a tryptophan auxotroph, strain 800, prepared from parent 6 by treatment with nitrosoguanidine. The recipients tested were all octane-negative, octanol-positive; the octanol-positive trait seems to be a character common to most fluorescent pseudomonads (5). Strain 800 served as donor to four of the recipients tested—though at low frequency. The preferred recipient, the phenol-positive strain U (PpG8) showed a frequency of about 10^{-7}/donor; the next-highest frequency was observed with the octane-deleted, CAM-positive *P. oleovorans* strain 801—the preparation of these strains are shown in Table 1. The octane-cured strain 968 and the wild-type Cam$^+$ strain, PpG1, were poor recipients. Again the *P. aeruginosa* strain, a10 (Holloway), was not found to serve as recipient; previously, we were unable to observe transfer of the camphor plasmid to this strain (10).

As we have shown in other experiments, plasmid transfer frequencies depend on the donor, the recipient, and the plasmid (10–12). Further experiments have indicated that high-frequency donor strains for the octane plasmid from *P. oleovorans* can be prepared; these will be the subject of future publications.

Regulation of octane plasmid expression

A study of the growth characteristics of Cam$^+$Oct$^+$Ocl$^-$ exconjugants of *P. oleovorans* has shed some light on the genetic regulation and expression of the octane plasmid. Wild-type *P. oleovorans* cells grow well with either octane or octanol as the sole source of carbon. A mixture of the two supports growth equally. The Oct^{del}Ocl$^+$ segregant, 968, however, does not grow with octane, but grows well with either octanol or a mixture of octane and octanol. In contrast, the Cam$^+$Oct$^+$-Ocl$^-$ exconjugant grows well with octane as the sole source of carbon, but not with octanol, and small quantities of octanol severely inhibit growth on octane as the sole carbon source. Growth inhibition did not occur if octanol was added to growth media with succinate or glucose as carbon source. Thus, it appears that octanol acts as a potent corepressor of

TABLE 4. *In presence of camphor plasmid, octanol inhibition of octane phenotype*

Carbon source		Strain	6	968	802
		cam	−	−	+
		oct	+	−	+
Octane	Octanol	*ocl*	+	+	−
*ml/10ml**				*Growth†*	
1	0		3+	−	3+
1	0.5		3+	3+	−
0	0.5		3+	3+	−
1	0.075		3+		−
1	0.050		3+		+
1	0.025		3+		2+

* PAS medium, growth for 24 hr at 30°.

† A_{660} = <0.2, −; →0.5, +; →1, 2+; >1, 3+.

the inducible ω-hydroxylase system, and possibly of other enzymes specified by the octane plasmid. The wild-type organism or the Oct^{del}Ocl$^+$ segregants would appear to grow well with an octane–octanol mixture, because even if octanol repressed induction of the plasmid-specified enzymes, it would lead to the synthesis of the duplicate octanol dehydrogenase whose genes appear to be chromosomal.

Finally, the size and information content of the octane plasmid is unknown. The capability of the Oct^{del} segregants to grow on octanol as the sole source of carbon suggested initially that the only genes present on the plasmids are those specifying the three ω-hydroxylase components. However, growth on octane, not octanol, under conditions where octanol dehydrogenase induction was inhibited by the camphor plasmid, suggests that the octane plasmid must also carry a octanol dehydrogenase gene under octane regulation. This finding raises the interesting question of how many of the octane catabolic enzymes are specified by both the plasmid-borne and chromosomal genes. Since most *Pseudomonas* strains grow on octanol, and since the octanol-degrading enzymes appear to be governed by chromosomal genes, the plasmid-borne genes for the synthesis of octanol-degrading enzymes are presumably redundant. It may be possible to determine the number of such genes in the octane plasmid by isolation of mutants in the Oct^{del} segregants that are octanol-, octanaldehyde-, or octanoate-negative. These mutants could then be used as recipients and the frequency of transfer of the octane plasmid could be determined. The absence of Oct$^+$ exconjugants from crosses with a particular mutant would indicate that the corresponding gene is not borne on the plasmid.

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