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A Transmissible Plasmid Controlling Camphor Oxidation in *Pseudomonas putida*

(peripheral metabolism/bacterial/episome/terpenes/genetics)

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ABSTRACT **Earlier papers demonstrated an extensive genetic exchange among fluorescent Pseudomonads; this one documents for genes specifying enzymes of peripheral dissimilation an extrachromosomal array, segregation, and frequent interstrain transfer. An hypothesis is presented of a general mechanism for the formation and maintenance of metabolic diversity. The example used, the path of oxidative cleavage of the carbocyclic rings of the bicyclic monoterpene D- and L-camphor, terminates in acetate release and isobutyrate chain debranching. By transduction, two gene linkage groups are shown for the reactions before and after isobutyrate. The group for reactions before isobutyrate is plasmid borne, cotransferable by conjugation, mitomycin curable, and shows a higher segregation rate from cells that are multiplasmid rather than carrying a single plasmid. The genes that code for isobutyrate and essential anaplerotic and amphibolic metabolism are chromosomal. By conjugation plasmid-borne genes are transferred at a higher frequency than are chromosomal, and are transferred in homologous crosses more frequently than between heterologous species. Most isobutyrate-positive fluorescent pseudomonad strains will accept and express the camphor plasmid.**

The three purposes of this paper are: (*i*) to record the genetic and chemical properties of a terpene dissimilation pathway including an extrachromosomal gene array in the fluorescent pseudomonad, PpG1. Previously communicated were Rheinwald's thesis (1), a brief review‡, and a resumé (2); (*ii*) to illustrate the interstrain transfer of plasmid-borne genes among fluorescent pseudomonad species and to suggest a workable nomenclature; and (*iii*) to present a mechanism for the formation and maintenance of diversity among organisms with limited genomes. The basic concepts without details were outlined earlier (3, 4).

The broad nutritional and metabolic potential of these bacteria is amply documented; partial definition of the genetic organization (5, 6) and regulation (6, 7) of the biosynthetic and dissimilatory processes has appeared. Selected strains have provided the methods and quantitation to define the enzymes and prosthetic groups functional in direct oxygenation, ring cleavage, and chain-debranching processes. Fig. 1 outlines, for a single terpene pathway (6, 8, 9), the essential facts required to follow the chemical intermediates, the metabolic pathways, the enzymes, and the definition of phenotypes and genotypes presented.

The demonstration of multienzyme oxygenase systems has provided forceful tools for the analysis of electron transport and oxygen reduction in chemical–physical terms—especially in the form of pure proteins of the unique substrate–oxygen binding cytochrome P450 (10), an iron sulfide redox protein (10, 11)—that permitted a recognition of the catalytic reaction cycles including the intermediate states of the prosthetic groups (8, 12). The chemical advance, with assays, has pinpointed mutant loci to define the genetic organization of the peripheral metabolic processes, to clarify genetic homologies between *P. aeruginosa* and the *P. putida fluorescens* group (13, 14), and to foster as well an improved communication among a growing group of investigators (15). In turn, the genetically prepared strains have allowed the selective labeling of the prosthetic groups of metallo active sites with isotopes and analogues and, thus, through physical and chemical measurements, to foster an understanding of structure and mechanism (11, 16).

2 Years after our discovery and elaboration of a conjugation system (1) in *Pseudomonas putida*, with demonstration of the clustering of genes controlling camphor oxidation on a plasmid, we record here the evidence accumulated with several fluorescent pseudomonad species upon which to base more extensive reports on the variety, transfer, and regulation of plasmids specifying diverse metabolic process (17–19).

MATERIALS AND METHODS

Bacterial Strains. Table 1 lists the parent (wild type) strains used with source, reference to earlier authors' work, and ATCC submission numbers. Urbana retention numbers PpG, PaG are Genus, P = *Pseudomonas;* species, p = *putida*, a = *aeruginosa*, etc.; G = Gunsalus collection. Table 2 summarizes the mutant numbers, with gene and phenotype designation, parent, and preparation. The "mandelate genes," Md1, are derived from Stanier strain A3.12 (PpG3) (20, 21); camphor pathway genes are from strain PpG1 (22, 23), with the main chemical and genetic experiments conducted in the PpG1 cell background. The *Pseudomonas aeruginosa* strains selected, PAT (15) and 9027 (Campbell *et al.*, ref. 24), are effective recipients, then donors, of the "CAM plasmid," e.g., a924 and a929.

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‡ Chakrabarty, A. M., Rheinwald, J. G. & Gunsalus, I. C., ASM Meeting, Minneapolis, May 1971, Symposium on Hydrocarbons: Genetic Organization of Oxygenases.

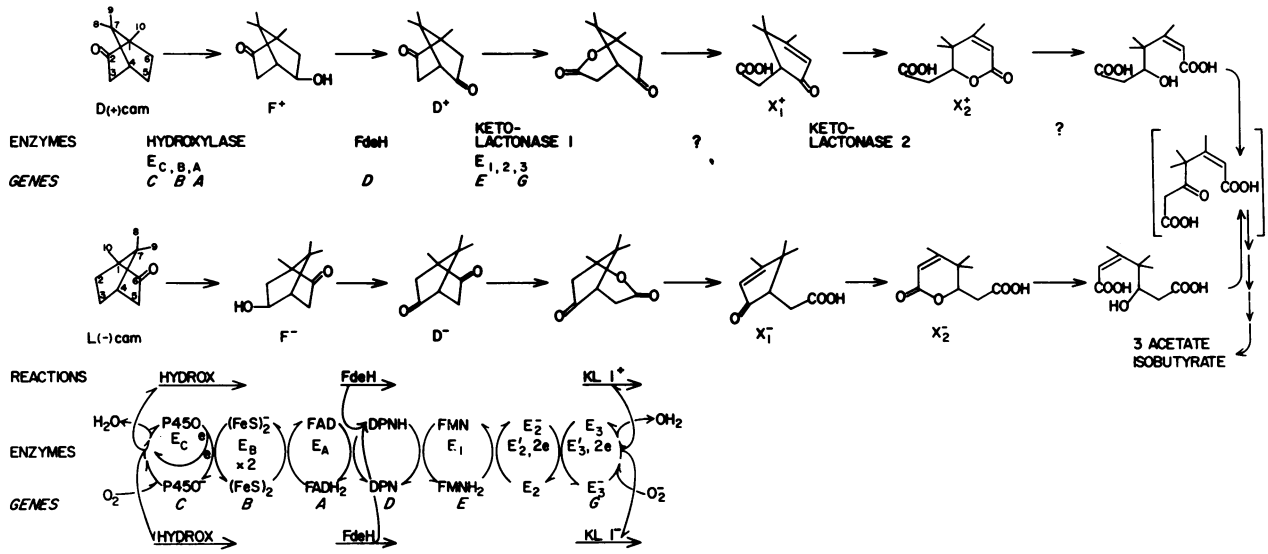

FIG. 1. D- and L-Camphor oxidation pathway in *Pseudomonas putida*.

Point Mutants for the carbon source, D-camphor, were selected after treatment of a stationary PpG1 (L-broth) culture with 100 μg/ml of (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) in citrate buffer, pH 6, to about 0.1% survivors, followed by replica plating for selection (23); spontaneous mutants were isolated in the presence of 5 mM D-camphor by penicillin-D-cycloserine selection (25).

Media and Culture Conditions. All cells were grown at 30° in L-broth, (26) and cells were harvested in early stationary phase. To select mutants and exconjugants, a minimal salt medium (4) was used with carbon sources at 5 or 10 mM plus agar, 1.5%. Cells for enzyme assay were from liquid minimal media containing 15 mM L-glutamate and 5 mM D-camphor, grown to late-log phase on a rotary shaker and harvested by centrifugation.

Mitomycin C Curing of "plasmid-borne genes" used nearly toxic levels of mitomycin C in L-broth inoculated with about 10^5 cells per ml of early stationary cultures, followed by 48 hr of incubation on a rotary shaker. These cultures were diluted in saline, plated on complete media, and replicated to minimal, glucose, or D-camphor plates to score and select individual clones for the Cam$^-$ phenotype.

Transduction and Conjugation. Phage pf16 was used in generalized transduction by published methods (23). For conjugation, stationary L-broth cultures of donor and recipient, about 10^{10} cells per ml, were mixed together and 0.1-ml aliquots were plated immediately on selection plates and incubated for 48–72 hr. Donor cultures were diluted with saline before mixing to yield fewer than 1000 exconjugant clones per plate (1).

Enzyme Assays. Extracts, prepared from concentrated suspensions (100 mg of wet cells per ml) by sonication with a Branson model 110C oscillator followed by centrifugation for 1 hr at 4° and 20,000 rpm in a Sorvall SS-34 head, contained 10–20 mg of protein per ml. *Hydroxylase* components E_A, E_B, and E_C (see Fig. 1) were assayed by measurement of substrate-dependent DPNH oxidation at 340 nm (10, 27); FdeH was measured by substrate-dependent DPN reduction according to Paisley (3, 27) and the *ketolactonase* 1-DPN dehydrogenase (E_1) by FMN-dependent DPNH oxidation according to Trudgill (28). Substrate-specific D-ketolactonase I activity (E'_2 and E'_3) was measured in a fixed-time product assay (9).

TABLE 1. *Wild-type Pseudomonas strains used*

Stock* *P-G*	*Pseudomonas*	ATCC*	SPD/BH†	Ref.
p 1	*putida, A*	17,453	77	22, 23
2	*putida, A*	23,287	—	23
3	*putida, A*	12,633	90	23
72	*putida, B*	17,470	96	20
*f*45	*fluorescens, E*	17,419	41	20
*a*10	*aeruginosa*	15,692	/PAO	15, 37
*a*12	*aeruginosa*	15,691	/PAT	15, 37
*a*13	*aeruginosa*	9,027	JJRC	24, 34

* *P–G Pseudomonas* Gunsalus collection, −, species (1, 19). ATCC, American Type Culture Collection.

† SPD = Stanier–Palleroni–Doudoroff opus (20); BH = Bruce Holloway (15); JJRC = J. J. R. Campbell (24).

RESULTS

Mutant Phenotypes. Table 3 lists by growth phenotype representative Cam$^-$ point and deletion mutants derived from PpG1—an additional 100$^+$ strains are characterized in (1). For convenience, the strains are considered in three groups: *I*, all retain the capacity to grow on the first acidic product of camphor oxidation X_1; *II* grow on isobutyrate, and *III* on neither, although they grow on succinate. Some strains among the group II mutants, and those of group I that do not grow on intermediates before the diketone, D, grow on L-camphor. These retain the stereospecific substrate reactive enzymes of ketolactonase 1 and 2, which differentiate between (+) and (−)-camphor (9)—for example, strains 557 and 566. In contrast, a single substrate-reactive hydroxylase protein $P450_{cam}$ catalyzes the exomethylene hydroxyl-

TABLE 2. *Pseudomonas strains used in genetic studies*

Stock P-G no.	Genotype	Phenotype	Derivation: Parent	Derivation: Treated†/Ref.
1	wt/CAM*	wt,Cam$^+$	-	Camphor, 22 Enrichment
273	trpB615/CAM	Trp$^-$,Cam$^+$	1	PC
327	trpD631/CAM	Trp$^-$,Str^r,Cam$^+$	1	NG, 38
799	trpB615,str-141/CAM	Trp$^-$,Str^r,Cam$^+$	273	S, 38
953	argH684/CAM	Arg$^-$,Cam$^+$	1	PC
841	met-608/CAM^d	Met$^-$,Cam$^-$	1	NG
571	/CAM^d	Cam$^-$	1	PC
572	/CAM^d	Cam$^-$	1	PC
543	/CAMcamC100	Cam$^-$	1	NG
797	str-143/CAM^d	Str^r,Cam$^-$	571	S
798	str-144/CAMcamC100	Str^r,Cam$^-$	543	S
2	wt/CAM	wt,Cam$^+$,Mdl$^-$	1	S, 23
3	wt/	wt,Mdl$^+$,Cam$^-$	-	Lactate, 20 Enrichment
822	/MDL	wt,Mdl$^+$	3 × 2†	T(h2), 21
927	trpD633,str-601 /MDLtrp(ABD)$^+$	Trp$^+$,Str^r,Mdl$^+$	822 x 898	C, Str CS, 32
a12	wt/	wt,Cam$^-$	-	37
a13	wt/	wt,Cam$^-$	-	24, 34
a924	/CAM	wt,Cam$^+$	273 x a12	C, aux CS
a928	/CAM	wt,Cam$^+$	273 x a13	C, aux CS
a168	phe-700/	Phe$^-$,Cam$^-$	a13	NG §
a929	phe-700/CAM	Phe$^-$,Cam$^+$	273 x a168	C, aux CS

* CAM, MDL – Plasmid designation; d = deleted (cured strains); MDL = pfdm defective phage with mandelate genes (1, 23); Cam = camphor; Mdl = mandelate.

† Crosses: →, transduction; x, conjugation: chromosomal loci precede/plasmid follow. T(h2) = transduction, h2 host range (23); C = conjugation; CS = counterselection.

‡ *NG* = nitrosoguanidine; *S* = spontaneous; *PC* = penicillin–cycloserine; *Str* = streptomycin.

§ Kindly donated by J. J. R. Campbell. // wt Cam$^+$ = 1, 2; Cam$^-$ = 3, a12, a13, Table 1.

TABLE 3. *Camphor mutant genes and growth phenotypes*

Stock no.	Genotype	Phenotype	Carbon sources: D-Cam	F	D	X_1	Ibu	Prp	Suc
1†	wt/CAM	wt,Cam$^+$	+†	+	+	+	+	+	+
544	/CAMcamA101	OH,E_A^-	-						
545	/CAMcamB102	OH,E_B^-	-						
543	/CAMcamC100	OH,E_C^-	-				(ALL +)		
552	/CAMcamD120	FdeH$^-$	-						
553	/CAMcamD121	FdeH$^-$	-						
557†	/CAMcamG133	KL,1$^-$	-						
556	/CAMcam-132	KL,1$^-$	-						
566†	/CAMcam-206	Z	-						
560	/CAMcam-200	Z	-				(ALL -)		
568	/CAMcam-208	Z	-						
571	/CAM^d		-						
572	/CAM^d		-						
575	ibu-100/CAM		-						
577	ibu-102/CAM		-						

* All from 1 by nitrosoguanidine, except 571 and 572.

† 1, 557, 566 L-(–)-Camphor +. Trivial names of compounds and enzymes, Fig. 1. D-Cam = (–)-camphor. F = 5-*exo*-hydroxycamphor; D = 2, 5-diketocamphane; X_1 = cyclopentenone acetic acid; Ibu = isobutyrate; Prp = propionate; Suc = succinate; OH$^-$ = Lack hydroxylase enzymes E_A, E_B, E_C, etc.; KL = ketolactonase; FdeH = F dehydrogenase; Z = reactions, enzymes unresolved.

ation of both (+) and (–)-camphor and their 1, 2 lactones (10, 11). As shown in Fig. 1, the proteins and gene designations are clear for the first three reactions, i.e., three hydroxylase genes *camABC*, the alcohol dehydrogenase *camD;* the DPNH dehydrogenase of ketolactonase 1, *camE*, and the D-(+)-camphor specific ketolactonase protein *camG*. The intermediate compounds, reactions, and enzymes remain to be clarified.

Transductional Linkage of cam Genes. Phage grown on the Cam$^+$ parent restores this phenotype to group I and II mutants at a frequency of about 10^{-7} per phage; for group III and auxotrophic mutants, the frequencies are near 10^{-6}. Spontaneous or penicillin–cycloserine-selected mutants are known to carry a high frequency of deletions (1). They do not transduce to Cam$^+$ or serve as donors to Cam$^-$ point mutants, nor do they revert to Cam$^+$ on mutagenesis. They are thus tentatively listed as deletants, CAM^d.

Table 4 shows the close linkage among group I and II genes; neither is linked to group III. Three levels, all above 50%, are observed—that is, *camA*, *B*, *C*, and *D* $\geq$ 0.9; *A* to *G* and *A*, *B*, or *C* to midpathway cistrons to 0.5 and 0.6 and among the midpathway loci, including *G* > 0.85. Fine-structure mapping is in progress.

Spontaneous and Mitomycin C Curing. Spontaneous Cam$^-$ mutants arise from PpG1 at frequencies around 10^{-4} per cell per division (1) and do not revert, i.e., $<10^{-10}$. All lack the early enzymes of the camphor pathway and act neither as recipients nor donors in transduction. Mitomycin C when added to L-broth cultures at nearly growth-inhibiting levels enhances the Cam$^-$ appearance in some strains, approaching a rate of 1 per cell per division. The mitomycin C sensitivity level is characteristic of the wild-type strain; the PpG1, *trpB615* mutant, 273, is unaffected by 10 μg/ml of mitomycin C, but 25 or 50 μg/ml yields Cam$^-$ cells in excess of 0.9 per cell per generation. The two Cam$^+$ *P. aeruginosa* exconjugants a924 and a929, derived from a12 and a13, are inhibited by 25 μg/ml of mitomycin, but 10 μg/ml produces Cam$^-$ progeny at frequencies of 0.8 and 0.94 per cell per generation. All the cured strains lack the camphor pathway enzyme activities for which we have assays. That is, the Cam$^-$ PpG1 derivative, 572, and *aeruginosa* strains a12 and a13 contain less than 1 international enzyme unit/mg of protein, whereas PpG1 and the exconjugants a924 and a929 contain, in —μmol

TABLE 4. *Transductional linkages cam loci*

Donor/Recipient	*D120*	*G133*	*–200*	*–208*
	Cotransfer frequency × 100*			
	/F$^+$	/D$^+$	/X_1^+	
A101	98	60	52	57
B102	95		67	56
C100	93		55	61
D121			57	61
G133			87	90
–200	(X_1^-/L-Cam$^+$) →		87	94

* $\geq$200 clones scored; selected on F, D, or X_1. Cam$^-$, nonselected, *cam* and *ibu* unlinked.

TABLE 5. *Spontaneous segregation: Cam⁺ and Cam⁺Mdl⁺ cells*

Test strain preparation		Exconjugant curing rate	
Cross D x R	Selected	Sel	Cam⁻ %/generation
927 × 327	min Cam⁺	min Mdl⁺	5–10
a929 × 927	min Cam⁺	min Mdl⁺	1–5
a929 × 572	min Cam⁺	min Suc⁺	0.01*

* Parent segregation rate.

min^{-1} per mg of $protein^{-1}$ (international unit)— the hydroxylase enzymes $E_{A,B,C}$ at 15, 10, 45; E_1 70–80 and FdeH 10–20. These data suggest that mitomycin C interferes with CAM replication—indicative of an autonomy.

Transmissibility and Plasmid Nature of cam Gene Cluster. Many plasmids are transmissible, e.g., RTF (29), ColIV (30), and F′ (31) of the enterobacteria and pfdm of *Pseudomonas* (32). Thus, the mobility of the *cam* genes was compared to presumed chromosomal genes, including *ibu*, *trp*, *arg*, and *met*, and to Str^r. Two-factor plate matings with auxotrophic, or Str^r, counterselection also served to identify the recipients. Strain 273, (*trpB*Cam^+) was crossed with 798 and 799 (Str^r Cam^-). Selection for Trp^+Cam^+ gave exconjugants (or conjugatants, ref. 33) carrying the unselected Str^r marker. In the reverse cross, 799, (a Str^r variant of 273) × 572 and 573 (Str^sCam^-), the Trp^+Cam^+ exconjugants were Str^s—that is, *cam* was transferred, Str^s was not. The Cam^+ "plasmid" transfer frequencies were about 10^{-5} per donor, the chromosomal markers $<10^{-9}$ again indicating the lack of linkage.

Table 5 shows the effect of a second plasmid on transfer and on the stability of the exconjugates. The preparation of three strains carrying CAM, MDL, or only CAM include: in the first pair of crosses the exogenote/MDL*trp*$(ABD)^+$ in donor, then recipient, where selection on camphor minimal media requires the presence of the Trp^+ exogenote for survival. The third cross concerns *cam* transfer to a CAM^d recipient. The first, 927 × 327, yielded unstable exconjugants that segregate to Cam^- at 5–10% per generation, compared to the second, a929 × 927, of 1–5% per generation. These rates are as much as 10^5 higher than those observed in the parent strain or the single plasmid exconjugants from the third cross, a929 × 572. These data support the plasmid array of *cam* genes.

TABLE 6. *Conjunction transfer plasmid versus chromosomal*

Cross D x R	Genotypes* R	Selected	Transfer frequency × 10^8
273* × 544	/CAM*camA101*	Trp^+, D-Cam^+	200
273 × 560	/CAM*cam-200*	Trp^+, D-Cam^+	60
263 × 575	*ibu-100*/CAM	Trp^+, Ibu^+	<0.1
273 × 841	*met-608*/CAM^d	Trp^+, Met^+	<0.1
273 × 953	*argH684*/CAM	Trp^+, Arg^+	<0.1
575† × 544	/CAM*camA101*	D-Cam^+	200
575 × 560	/CAM*cam-200*	D-Cam^+	1000
575 × 577	*ibu-102*/CAM	Ibu^+	<0.1
544‡ × 560	/CAM*cam-200*	D-Cam^+	10

* D273 = *trpB615*/CAM; †575, *ibu-100*/CAM; ‡544, /CAM-*camA101*.

TABLE 7. *Conjugation of CAM plasmids: Intra- versus inter-species*

Recipient strain	Ibu	Donor 273	Donor a929
		Frequency/donor × 10^6	
572	+	2000	100
3	+	10	3
72	+	2000	100
f45	+	500	100
a13	+	0.2	5
a12	+	0.4	0.001
a10	+	<0.001	<0.001

Chromosomal Versus Plasmid Transfer. Table 6 compares three mutant donors, *trpB*, *ibu*⁻, and *camA*, with selection for Cam^+, Ibu^+, or protophs by use of Trp^+ or Ibu^+ counterselection. The *cam* transfer ranged from 10^{-5} to 10^{-6} per donor, save for the cross of the two *cam* mutants, in which the frequency was reduced to 10^{-7}. The chromosomal markers were not transferred above a frequency of 10^{-9} per donor. Conditions for the transfer of chromosomal markers have been observed (32); further data will be the topic of a later manuscript.

Recipient Range of CAM Plasmids. The evidence of genetic homology among *Pseudomonas putida*, *fluorescens*, and *aeruginosa* (13, 14, 34) was extended to the CAM plasmid. Table 7 shows the results of crosses by two representative donors 273, *trpB* and a929, *phe* (respectively derived from strains 1 and a13) with protoph counterselection. Per donor cell, the two differ about 20-fold favoring the *P. putida* donor, except the homologous recipient, a13, was favored by 25-fold with the a929 donor. The transfer frequency clearly indicates a role of both donor and recipient in the effectiveness of both intra- and inter-strain conjugation.

DISCUSSION

The data presented, which demonstrate a plasmid role in the genetic control of peripheral metabolism, suggest experiments and potential that extend beyond the CAM plasmid to the general problem of diversity and gene economy.

The closely linked loci specifying inducible camphor dissimilation enzymes provides a third example, extending the previous recognition of a mandelate dissimilatory gene cluster (23) and three linkage groups in tryptophan biosynthesis (23, 34) in *Pseudomonas putida*. The three cistrons, specifying hydroxylase component proteins (10, 11) plus the alcohol dehydrogenase (FdeH) cistron under coordinate regulation (4), is unusual only in the appearance of relaxed inducer specificity (4) and is consistent with regulation at the transcriptional level. The location of the *ibu* genes, most likely chromosomal, and the degree of clustering can now also be defined, and the apparent inability to transfer the entire set of genes specifying the camphor pathway by transduction can be defined as well.

The replicative autonomy and plasmid nature of the *cam*

gene cluster seem assured on several lines of evidence: these include the spontaneous and mitomycin C-induced formation of Cam$^-$ mutants lacking the entire complement of camphor pathway enzymes, the transmissibility of the entire *cam* gene cluster by conjugation without chromosomal genes, and the incompatibility of *cam* genes with the known extrachromosomal element carrying/MDL...*trp*(ABD)$^+$. The incompatibility could derive from a competition for the same replicative membrane attachment site, or from poor distribution of the nonselected plasmids to daughter cells. Analogous incompatibility is known among plasmids in *E. coli* and *Staphylococcus aureus* (35, 36).

The evolutionary significance of tightly clustered genes specifying peripheral metabolic traits associated with specialized transfer is pertinent to an evaluation of the nutritional versatility of fluorescent pseudomonads, as is the maintenance and extrachromosomal array of the *cam* gene cluster in *Pseudomonas putida* PpG1. The initial source of the plasmid and means of attaining replicative autonomy, although obscure, are reminiscent of the phage pf16h2-mediated transfer of the mandelate gene cluster with *trp*(ABD)$^+$ from PpG3 to PpG1, where the survival of the recipient on tryptophan-free medium requires retention and replication of the exogenote, since lack of homology with the chromosome appear to preclude integration. The genetic organization of the mandelate genes in PpG3 is unusual (37) relative to other *P. putida* and *P. aeruginosa* strains, and will also merit further attention.

Further analyses of peripheral pathways in dissimilatory metabolism and their genetic bases have been initiated (17, 19) and will be extended also as a step in the eventual analysis of the general significance of the type of genetic organization and regulation reported here.

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