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Transmissible Plasmid Coding Early Enzymes of Naphthalene Oxidation in *Pseudomonas putida*

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The capacity of *Pseudomonas putida* PpG7 (ATCC 17,485) to grow on naphthalene, phenotype Nah^+, is lost spontaneously, and the frequency is increased by treatment with mitomycin C. The Nah^+ growth character can be transferred to cured or heterologous fluorescent pseudomonads lacking this capacity by conjugation, or between phage pf16-sensitive strains by transduction. After mutagenesis, strains can be selected with increased donor capacity in conjugation. Clones which use naphthalene grow on salicylate and carry catechol 2,3-oxygenase, the initial enzyme of the aromatic α-keto acid pathway, whereas cured strains grow neither on salicylate nor naphthalene and lack catechol 2,3-oxygenase, but retain catechol 1,2-oxygenase and the aromatic β-keto adipate pathway enzymes.

The diversity of carbon sources available to *Pseudomonas* species encouraged extensive biochemical investigation of their oxidative pathways (6, 7, 11, 15-19). More recent studies revealed conditions for genetic exchange by transduction (3, 4, 10) and by conjugation (4, 19) and permitted the analysis of the organization and regulation of both biosynthetic (3, 6, 10) and catabolic processes (9, 11, 15, 16, 19). Notable among the latter are conversion of the terpene camphor to acetate plus isobutyrate (1, 11, 13) and cleavage of the aromatic (benzenoid) nucleus (15, 18).

Gene transfer between selected organisms by transduction with phage pf16 (3, 10) or the host range mutant pf16h2 (3) was accomplished with the strains of *Pseudomonas putida* PpG1, 2, and 3, and between *P. aeruginosa* and *P. putida* with phage pf20 (5). We observed a spontaneous and a mutagen-induced loss of the camphor (Cam^+) growth phenotype from strain PpG1 and subsequently initiated a detailed investigation of the genetic basis for camphor metabolism (J. G. Rheinwald, M.S. thesis, University of Illinois, Urbana, 1970). Linkages among the genes governing camphor oxidation were shown by transduction and by curing the entire Cam^+ phenotype by growth in the presence of mitomycin C. The Cam^+ phenotype was also transferred to Cam^- strains by conjugation, and the genes were found to reside on a transmissible plasmid (16). The genes which code for the enzymes of the mandelate and aromatic oxidation pathways in strains PpG1 and PpG3 show close linkage by transduction, but evidence for their plasmid array is lacking (4, 12, 18).

The experience with camphor dissimilation suggested that plasmids may serve an important role in the genetic development of metabolic diversity and led us to examine other pathways for the occurrence of plasmid-borne genes. Naphthalene oxidation represented an attractive case because (i) after a few steps, the pathway converges at catechol, with the oxidative routes for many aromatic compounds, including salicylate, phenol, cresol, benzoate, anthranilate, and benzene (6, 7), and (ii) naphthalene-positive strains generally possess both the β-keto adipate and α-keto acid pathways for catechol utilization (7, 9, 19). A detailed study of naphthalene metabolism may therefore suggest fruitful directions for future work on the genetic organization of converging, possibly redundant, pathways.

This paper concerns the genetic basis of naphthalene oxidation in two strains of *P. putida*.

MATERIALS AND METHODS

Bacteria and phage. Three *P. putida* strains isolated from separate sources were used. Their properties are listed in Table 1 together with those for the derived strains prepared for the experiments of this paper. The two strains capable of growing on naphthalene PpG7 (ATCC 17,485) and PpG63 (ATCC 17,484) were obtained from the Stanier collection

TABLE 1. *Pseudomonas putida strains and their origins*

Stock PpG no.[a]	Genotype[b]	Phenotype	Derivation	
			Parent	Treatment (reference)[c]
1	wt/CAM	Cam^+		Enrichment (1, 17) ATCC 17,453; S77
273[d]	*trpB615*/CAM^d	Trp^-Cam^+	1	S, PC (5, 16)
572	/CAM^d	Cam^-	1	S, PC (5, 16)
701	*str-512*/CAM^d	$Str^r \cdot Cam^-$	572	NG
390	/CAM^d·NAH	Cam^-Nah^+	379 × 572[e]	C
391	/CAM^d·NAH	Cam^-Nah^+	379 → 572[e]	pf16
7	wt/NAH	Nah^+		Enrichment (17) ATCC 17,485; S111
376	/NAH^d	Nah^-	7	MC
377	/NAH^d	Nah^-	7	S
378	*leu-801*/NAH	Leu^-Nah^+	7	NG
379[d]	*leu-801*/NAH^d	Leu^-Nah^+	378	NG
63	wt	Nah^+		Enrichment (17) ATCC 17,483; S110
380	*leu-901*	Leu^-Nah^+	63	NG

[a] PpG, Urbana collection number. Genus *Pseudomonas* (P); species *putida* (p); Gunsalus (G, reference 16). All strains used are *P. putida* biotypes biotypes A or B; thus, stock numbers only are used in the text.

[b] Abbreviations: wt, wild type; superscript d (e.g., CAM^d), indicates a deletion (cured strains, reference 16).

[c] Abbreviations: S, spontaneous; MC, mitomycin C; NG, nitrosoguanidine; PC, penicillin-cycloserine (16); C, conjugation. S77, S111, and S110 are stock collection numbers in reference to the Stanier opus (17).

[d] High-frequency donor for plasmid markers in conjugation. Cam and NAH are plasmid designations.

[e] Crosses. Symbols: → transduction; ×, conjugation. Donor is first number. Chromosomal loci precede → or ×, plasmids follow.

(17); the camphor-positive strain PpG1 (ATCC 17,453) was isolated in Urbana by enrichment and used extensively in chemical and genetic studies (1, 11). In the Stanier, Palleroni, and Doudoroff study (17), the naphthalene-positive strains were numbered 111 (PpG7) and 110 (PpG63), the camphor strain PpG1 was numbered 77; strains PpG1 and PpG7 were classed as *P. putida* biotype A and PpG63 as biotype B (17). The phage pf16, isolated by Chakrabarty (10), was used in both transduction and phage sensitivity tests.

General cultural conditions. Unless specified otherwise, the procedures employed throughout this work were as described earlier (10).

Genetics. Mutants were prepared by treating cell suspensions with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) at 25 µg/ml in 50 mM citrate buffer at pH 6 and at 30 C for 30 min, as adapted from Adelberg (see 8). After mitomycin C treatment and after spontaneous segregation or mutagenesis, cells were selected and scored for phenotype.

Transduction was accomplished with phage pf16 in the phage-sensitive strain PpG1 by published procedures (10).

Conjugation. Plate and patch mating techniques were used for quantitation and for exploratory screening, respectively. Plate mating employed both donor and recipient grown at 30 C in liquid Luria (L) medium (14). The L-broth, 5 ml in 18- by 150-mm test tubes, was inoculated with actively growing cells, and the donors were incubated in stationary culture to about 5×10^8 cells per ml. The recipients were incubated with agitation at 200 rpm in a shaker to late log phase, about 5×10^9 cells per ml. The recipients were collected by centrifugation and suspended in equal volumes of saline, and 0.1 ml was plated together with 0.1 ml of donor on the surface of appropriate selection plates. When necessary, the donor strains were diluted in L-broth to give approximately 150 exconjugants per plate. The donor parent was eliminated by selection against auxotrophic markers and, in a few instances, streptomycin was added at 250 µg/ml to eliminate the streptomycin-sensitive parent. In the auxotrophic selection, the donor markers used reverted to prototrophy at frequencies of $<10^{-9}$. Patch mating was used for a qualitative screening of large numbers of clones for transfer of genetic material. Thirty individual clones were patched on each L-agar plate, incubated for 5 h, and replicated to appropriate selection plates on which had been spread 0.15 ml of the recipient culture prepared as described for the plate mating procedure.

Mitomycin C curing. The method of Rheinwald et al. (16) was used as follows. Cells (10^4 to 10^5) were inoculated into 1 ml of L-broth containing mitomycin C in 5-µg increments from 0 to 20 µg/ml and incubated with shaking for 36 h. Then, 0.1 ml was spread on L-agar plates and replicated to the appropriate test plates for scoring (Table 2).

Enzyme induction and assay. The organisms were grown on a basal salts medium (PAS) (13) with succinate at 10 mM with or without inducer at 2.5 mM using naphthalene, salicylate, catechol, benzoate, or phenol. Cells were harvested in logarithmic phase (5×10^8 per ml), and sonic extracts were prepared and assayed by previously standardized methods (9).

Catechol 1,2-oxygenase (EC 1.99.2.2) was estimated by the method developed by Hegeman (12). The activity is expressed as international units, micromoles of *cis*, *cis*-muconate produced per minute per milligram of protein based on $\epsilon_{mM}^{260\ nm} = 17$ (9). Catechol 2,3-oxygenase (EC 1.99.2a) activity was

TABLE 2. *Curing of Nah^+ growth phenotype by mitomycin C*

Strain	Surviving cells cured (%)				
	0[a]	5	10	15	20
7	0.2	2	10[b]	No growth	
63	<0.05	<0.05	<0.05	No growth	

[a] Micrograms of mitomycin C per milliliter.

[b] Also Sal^-.

assayed according to Feist and Hegeman (9) and expressed in the same units based on the micromoles of 2-hydroxy-muconic semialdehyde formed and the $\epsilon_{mM}^{375\ nm} = 30$.

Transfer of CAM plasmid to Nah^+ and Nah^- strains. The strain 273 (Trp^-Cam^+) a tryptophan auxotroph in a chromosomal cistron, bearing the camphor (CAM) plasmid, also carries a high-frequency donor capacity in conjugation (16). We tested this strain for transfer of CAM to the Nah^- strains 376 and 377 and to the Nah^+ parents 7 and 63. Table 3 (column 1) shows a thousandfold lower transfer of CAM to the parent 7 than to the cured strains 376 and 377 or the "noncurable" wild type 63. This negative effect resembles earlier observations of Cam^+ transfer to strains bearing the CAM plasmid, but the CAM plasmid is unable to grow on camphor because of a point mutations (16) and may arise from the presence of an "NAH plasmid." The Nah^+ transfer to the cured and "plasmid-free" wild type 63 of 10^{-2} frequency per donor (Table 3) approaches the best frequencies for Cam^+ transfer observed in homologous strain conjugation (16). The noncurability of Nah^+ in strain 63 is reminiscent of the mandelate genes of PpG3 (15); the high-frequency recipient activity for Cam^+, resembling the cured strains, is tentatively taken to suggest the lack of a plasmid. As mentioned earlier, this strain will require further analysis and will be the subject of a future publication. Its occurrence need not impede the analysis of the genetic and regulatory organization of strain 7, the topic of this paper.

The exconjugants from the first cross indicated in Table 3 of Cam^+- and Nah^+-bearing parents, i.e. 273 × 7, yielded on purification about 60% Cam^+Nah^+ and 40% Cam^+Nah^- phenotypes. The former segregated to Cam^- and Nah^- independently at approximately the same frequency per cell generation as the parent strains. The 40% Cam^+Nah^-, though unexpected, could derive from either of two events: the higher frequency, by 10^3, transfer to Nah^- segregants in the recipient population, or a forced segregation of an NAH plasmid at the time of CAM introduction. Further attention to this behavior is in progress.

TABLE 3. *Donor-recipient effects on conjugation*[a]

Recipient[b]		Donor no. and genotype		
No.	Genotype	273[c] *trpB615/* CAM	378 *leu-801/* NAH	379[c] *leu-801/* NAH
7	wt/NAH	55		
376	/NAD[d]	2	8	3
377	/NAH[d]	2	8	3
63[d]	wt·Nah^+	2		
572	/CAM[d]		8	3
701	*str-512*/CAM[d]			4^-

[a] Measures as frequency per donor × 10^{-n}, where n = number as indicated in columns.

[b] Selection on minimal media with energy source camphor 273; naphthalene 378, 379, and 701; for 701 also 250 μg of streptomycin per ml. Superscript d (e.g., NAH^d) indicates deletion (cured strains, reference 16). Abbreviation: wt, wild type.

[c] High-frequency plasmid donor.

[d] No evidence of plasmid.

Mode and frequency of NAH plasmid transfer. To complement the presumptive evidence of a plasmid-borne Nah^+ and Sal^+ genes in strain 7 we prepared a stable Leu^- auxotroph, PpG378, by nitrosoguanidine treatment and tested the donor capacity for the naphthalene growth phenotype, Nah^+. Minimal medium with naphthalene as carbon source was used to counterselect against this Leu^- parent. Three Nah^-Cam^- recipients were tested—the two segregants 376 and 377 prepared from strain 7 by spontaneous and mitomycin C curing and the spontaneous Cam^- segregant 572 from strain 1. In each case, though at very low frequency, we were able to select Nah^+ exconjugants (Table 3, center column). Since the analogous donor capacity for the CAM plasmid is variable over a wide range in derivatives of strain 1, we attempted to select a higher frequency NAH donor. In one attempt we treated the Leu^- strain 378 with NG and patch tested 1,000 clones for increased ability to transfer Nah^+ to strain 572. About 20 (2%) of the clones transferred this phenotype at increased frequency, approaching in effectiveness the CAM donor level of 273. The enhanced NAH transfer by one such donor, 379, is shown in the last column of Table 3. The same recipients used earlier received the NAH plasmid at similar frequencies. With the Str^r strain 701, the presence of streptomycin at 250 μg/ml as an additional counterselective agent against the Str^s donor 379 may have reduced the conjugation frequency somewhat.

An identical series of experiments was carried out with the second naphthalene-utilizing strain 63. A Leu^- mutant was isolated after retreatment with NG, and 1,000 clones were patch tested for Nah^+ transfer to strains 572 and 376. We did not detect the transfer of the naphthalene growth phenotype.

As mentioned in the introduction, phage pf16 provides a good transducing system for strain 1 and its derivatives. The phage is effective for markers in both biosynthetic and catabolic pathways in either extrachromosomal (plasmid) or chromosomal array. For genetic study on the naphthalene oxidation system, a well characterized strain in which transduction is possible would be most helpful. Therefore, twenty Nah^+ exconjugants, derived from the cross 379 × 572, were purified and tested for pf16 sensitivity. All were sensitive, as is characteristic of the parent strain 1, whereas strain 7 and its derivatives are pf16 resistant (see Table 1). The Nah^+ exconjugants in the strain 1 background are also curable of NAH and express the greater mitomycin C tolerance of strain 1 (16). For example, strain 390 on one passage with 15 μg of mitomycin per ml (about 16 generations) gave 50% Nah^- cells among the survivors. The phage pf16 when grown on 390 effected transduction of the Nah^+ phenotype to the recipient 572. The frequency, 10^{-7} per input phage, compared well to the 10^{-6} frequency for typical chromosomal markers. Interestingly, all the genes required for naphthalene oxidation and mobilization are transferable within the 10^8 dalton

genome of the transducing phage. Whether the genes are transferred by conjugation or by transduction, they are further transferrable by either procedure and remain in a form curable by mitomycin C treatment. Thus, the mobilization factor for conjugation is either independently present in the parent strains or is transferred by either method of gene transfer.

Naphthalene dissimilation pathway. The growth response of both parent and derived strains in respect to naphthalene and to simpler benzenoid compounds is shown in Table 4. The wild types 1 (17) and 7 (9) conform to earlier reports, and the phenotypes of the derived strains show that Nah and Sal are lost and gained together upon curing and upon crosses by both conjugation or transduction. In contrast, the capacity to grow on benzoate, Ben^+, and on phenol, Phl^+, in which the two wild types differ, is unaltered by these procedures. Thus, Nah^+ and Sal^+ appear to be linked and coded by a curable extrachromosomal element, whereas the noncurable Phl^+ and Ben^+ loci would appear to be carried on the chromosome.

Naphthalene is degraded via salicylate to catechol. The oxidation of catechol in strain 7 occurs by either of the two alternative pathways (9) termed, respectively, the β-keto adipate (ortho cleavage) and the aromatic α-keto acid (meta cleavage) pathways. These differ in the position of ring celavage, are initiated by different oxygenase enzymes, and are regulated by different inducers. The β-keto adipate pathway enzymes are induced by *cis, cis*-muconate, the product of catechol cleavage by the 1,2-oxygenase, whereas the aromatic α-keto acid pathway is initiated by a 2,3-oxygenase induced by the phenolic precursor of catechol (9).

Table 5 shows the enzyme induction patterns of the 1,2- and 2,3-oxygenases in the cells whose growth requirements are shown in Table 4. As reported by Feist and Hegeman (9), strain 7 expresses both catechol 1,2- and 2,3-oxygenases (Table 5). When

TABLE 4 *Growth phenotypes of NAH and NAH strains*[a]

Parent strain[b]		Growth substrate phenotype[c]					
No.	Genotype	Cam	Nah	Sal	Phl	Ben	Suc
7	wt/NAH	−	+	+	−	+	+
379	*leu-801*/NAH	−	+	+	−	+	+
376	/NAH[d]	−	−	−	−	+	+
377	/NAH[d]	−	−	−	−	+	+
1	wt/CAM	+	−	−	+	+	+
572	/CAM[d]	−	−	−	+	+	+
390	/CAM[d]·NAH	−	+	+	+	+	+
391	/CAM[d]·NAH	−	+	+	+	+	+

[a] Superscript d (e.g., NAH[d]) indicates deletion (cured strains, reference 16).

[b] Derived from parents 7 and 1 (see Tables 1 and 2). Abbreviation: wt, wild type.

[c] PAS medium (13) substrate at 10 mM, except naphthalene and phenol which were supplied in vapor phase. Cam, camphor; Nah, naphthalene; Sal, salicylate; Phl, phenol; Ben, benzoate; Suc, succinate.

TABLE 5. *Naphthalene plasmid and the gene coding catechol 2,3-oxygenase*[a]

Parent		Sp act × 10^2 (1,2/2,3-oxygenase)					
No.	Genotype[b]	Nah[c]	Sal	Phl	Ben	Cat	Suc
7	wt/NAH	16/36	15/35	0[d]/2	50/0	58/0	0/4
376	/NAH[d]	0/0	0/0	0/0	52/0	54/0	0/0
377	/NAH[d]	0/0	0/0	0/0	54/0	54/0	0/0
572	/CAM[d]	0/0	0/0	58/0	60/0	58/0	0/0
390	/CAM[d]·NAH	17/32	18/30	59/0	58/0	56/0	0/0
391	/CAM[d]·NAH	18/34	17/31	57/0	60/0	58/0	0/0

[a] For abbreviations, see footnotes to Table 4; Cat = catechol.

[b] Superscript d (e.g., NAH[d]) indicates deletion (cured strains, reference 16).

[c] Inducer: grown on PAS medium with 10 mM succinate plus inducers to about 2.5 mM.

[d] 0 = minimal detectable levels were <0.5 and 0.1, respectively, for the 1,2- and 2,3-oxygenases.

succinate serves as carbon source and either catechol or benzoate is present as inducer, only the catechol 1,2-oxygenase is formed, i.e., no 2,3-oxygenase.

Both naphthalene and salicylate will serve as primary carbon source for growth, and in their presence the catechol 2,3-oxygenase is formed. In our hands, the 1,2-oxygenase is present, although in reduced amounts—about ⅓ the level found in cells grown in the presence of benzoate or catechol. Presumably, the catechol 1,2-oxygenase and related enzymes are formed in the presence of naphthalene or salicylate because the inducer, *cis, cis*-muconate, is formed by the constitutive enzyme levels and serves to partially induce β-keto adipate pathway enzymes.

The Nah^- strains, from parent 7 or 1, which do grow on benzoate, catechol, and phenol, form the catechol 1,2-oxygenase, as shown earlier by Ornston (15) (table 5). In contrast, the Nah^+ exconjugant 390 formed in the cross 399 × 572 and the transductant 391, also prepared from 572, produce an inducible 2,3-oxygenase. The enzyme levels are comparable to the parent 7 from which the NAH plasmid was derived.

Each of these results is compatible with our conclusion that genes governing the conversion of naphthalene to catechol are carried on an NAH plasmid, whereas the coding for enzymes of the β-keto adipate pathway are not on the NAH plasmid and do not appear to be plasmid borne. It is possible that at least the initial enzyme in the aromatic α-keto acid pathway, the 2,3-oxygenase, is also present on the plasmid. Alternatively, the induction mechanism for this oxygenase may require the presence of the plasmid.

DISCUSSION

The genes coding for the enzymatic conversion of naphthalene via salicylate to catechol and aromatic ring cleavage via the 2,3-oxygenase are plasmid borne in *P. putida* strain PgG7. In contrast, no genetic evidence was

adduced for a plasmid array of these genes in strain PpG63, where they may be chromosomal. Current studies in our laboratory include physical demonstration for the presence of a plasmid in strain PpG7 and its absence in PpG63. The "plasmid" in PpG7, here termed NAH, is transmissible by conjugation or by transduction. Conjugation experiments included mostly plate matings. It was previously shown elsewhere that this type of cell-to-cell contact is necessary to obtain exconjugants (3). For genetic analysis by transduction, the plasmid was transferred to a cell background of PpGl origin where phage pf16 sensitivity is retained. The genes for the early steps in naphthalene dissimilation are closely linked. The linkage by transduction and the curability of the plasmid optimize this system for further genetic analysis, including detailed mapping of these genes which will follow improvement of the liquid mating system. It would also be worth checking the possibility of homology between the NAH and the SAL plasmids (2).

From this laboratory we have reported earlier that the genes coding for the oxidation pathways of camphor (16) and octane (A. M. Chakrabarty and I. C. Gunsalus, 1971, Genetics **68**:S10) are plasmid borne. It appears that plasmids play an important role in the evolution of metabolic diversity among the pseudomonads. The occurrence of these plasmids also bears important implications from a practical viewpoint, in promoting the rate and degree of cycling of natural and man-made organic residues.

Genetic analysis of strain PpG3 (PRS1) by transduction has been reported for several catabolic pathways. These studies also indicate gene clusters for enzymes of a particular catabolic pathway (4, 18); so far evidence of a plasmid is absent. After interstrain transduction of the mandelate gene cluster from PpG3 to PpG2, however, we were able to demonstrate a locus for chromosome mobilization (A. M. Chakrabarty and I. C. Gunsalus, Bacteriol. Proc., 1971, p. 46).

The second naphthalene degrading strain, *P. putida* PpG63, also contains inducible enzymes for both the β-keto-adipate and aromatic α-keto acid pathways (9). We were unable, however, to show extrachromosomal linkage. This may represent a case of insufficient detection or curing procedures. We prefer, however, the working hypothesis that the relevant genes are chromosomal in strain 63. If the latter be true, two evolutionary explanations are possible: either the ability to degrade naphthalene was of plasmid origin in both strains, but became integrated in PpG63, or vice versa. In strain PpG3, for example, there is evidence for genetic clustering of particular catabolic pathways, i.e. mandelate, catechol, etc., that are noncurable by procedures effective in related strains. This may have occurred because the relevant genes are integrated into the chromosome. This area is worthy of further work, in hope of understanding the short-range evolution of pseudomonad strains and of increasing basic operational knowledge of the number of pathways known to be carried and transferable via plasmids.

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