JOURNAL OF BACTERIOLOGY, Aug. 1975, p. 546-556

Vol. 123, No. 2
Printed in U.S.A.

Regulation of Alkane Oxidation in *Pseudomonas putida*

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Received for publication 18 April 1975

We have studied the appearance of whole-cell oxidizing activity for *n*-alkanes and their oxidation products in strains of *Pseudomonas putida* carrying the OCT plasmid. Our results indicate that the OCT plasmid codes for inducible alkane-hydroxylating and primary alcohol-dehydrogenating activities and that the chromosome codes for constitutive oxidizing activities for primary alcohols, aliphatic aldehydes, and fatty acids. Mutant isolation confirms the presence of an alcohol dehydrogenase locus on the OCT plasmid and indicates the presence of multiple alcohol and aldehyde dehydrogenase loci on the *P. putida* chromosome. Induction tests with various compounds indicate that inducer recognition has specificity for chain length and can be affected by the degree of oxidation of the carbon chain. Some inducers are neither growth nor respiration substrates. Growth tests with and without a gratuitous inducer indicate that undecane is not a growth substrate because it does not induce alkane hydroxylase activity. Using a growth test for determining induction of the plasmid alcohol dehydrogenase it is possible to show that heptane induces this activity in hydroxylase-negative mutants. This suggests that unoxidized alkane molecules are the physiological inducers of both plasmid activities.

Several strains of *Pseudomonas* are capable of growth on *n*-alkanes and oxidize these compounds by the following pathway: $R\text{-}CH_3 \rightarrow R\text{-}CH_2OH \rightarrow R\text{-}CHO \rightarrow R\text{-}COOH \rightarrow$ beta oxidation (1, 7, 10, 16-18). *Pseudomonas putida* strains grow on *n*-alkanes of 6 to 10 carbon atoms by virtue of the transmissible OCT plasmid (2, 5, 10). This substrate range is strain specific and is due to the capacity of OCT^+ *P. putida* strains to hydroxylate these alkanes to the corresponding primary alcohols (10).

Whole-cell alkane-oxidizing activity is inducible in *P. aeruginosa* strains (1, 16) as well as in other alkane-utilizing bacterial species (11). In *P. aeruginosa* 473 the effective inducers include alkane growth substrates, aliphatic diols, straight-chain diethers, and dicyclopropyl compounds (20, 22). There has, however, been no detailed analysis of the induction of alkane-oxidizing activity in *P. putida* strains.

This paper reports the results of a series of physiological and genetic experiments with alkane-utilizing *P. putida* strains designed to elucidate the nature of OCT plasmid-coded oxidizing activities, identify the inducers of these activities, and define the role that induction plays in determining growth on alkanes and their oxidation products.

MATERIALS AND METHODS

Bacterial strains. The *Pseudomonas* strains used are listed in Table 1.

Microbiological methods. Media, culture conditions, growth tests, and mutagenesis procedures are as described by Nieder and Shapiro (10).

Chemicals. These are generally as described by Nieder and Shapiro (10). *n*-Heptane from Fisher Chemical Co. ("Spectranalyzed") was used as a carbon source in some experiments, but induction experiments were performed with *n*-heptane (99.9%) from Chemical Samples Co. Dicyclopropyl ketone (DCPK) and dicyclopropryl methanol (DCPM) were purchased from K and K Laboratories, Inc. Rifampin and chloramphenicol were obtained from Sigma Chemical Co. [1-^{14}C]Nonane (3.67 mCi/mmol) was purchased from ICN Pharmaceuticals, Inc. and diluted in cold nonane (99.9%) from Chemical Samples Co. Silicic acid (silicAR cc-7) was purchased from Mallinckrodt.

Silicic acid chromatography performed after the experiments reported here revealed that approximately 3% of the radioactivity in our labeled nonane is retained on the column after elution with petroleum ether. These counts could be eluted with methanol and presumably represent contamination by oxidized substances. It is possible that some of the background level of nonane-oxidizing activity detected in uninduced and OCT^- cultures is due to the oxidation of this contaminating material.

TABLE 1. *Bacterial strains*[a]

Strain	Genotype	Source
P. aeruginosa		
PAS48	PAO *leu*⁻ (CAM-OCT)	A. Chakrabarty
P. putida		
PpG1	Wild type (no plasmid)	A. Chakrabarty
PpG6	Wild type (OCT⁺)	I. C. Gunsalus
PpG1737	*alcA*⁻ *str*	I. C. Gunsalus
PpS4	OCT⁻ (PpG6 cured by mitomycin)	I. C. Gunsalus
PpS5	*met*⁻ (OCT⁺)	A. Chakrabarty
PpS69	*alcA*⁻*ald*⁻*str*	NTG mutant of PpG1737
PpS70	*alcA*⁻ *str* (OCT⁺)	PpS5 × PpG1737
PpS76	*alcA*⁻	NTG mutant of PpG1
PpS81	*alcA*⁻	NTG mutant of PpG1
PpS84	*alcA*⁻ *str* (OCT *alk*⁻ *alcO*⁺)	NTG mutant of PpS70
PpS88	*alcA*⁻ *str* (OCT *alk*⁻ *alcO*⁺)	NTG mutant of PpS70
PpS124	*alcA*⁺ *str*⁺ (CAM-OCT⁺)	PAS48 × PpG1
PpS125	*alcA*⁻ *str*⁺ (CAM-OCT⁺)	PAS48 × PpS76
PpS126	*alcA*⁻ *str*⁺ (CAM-OCT⁺)	PAS48 × PpS81
PpS181	*alcA*⁻ *str*⁺ (CAM-OCT *alk*⁻ *alcO*⁺)	NTG mutant of PpS125
PpS191	*alcA*⁻ *str*⁺ (CAM-OCT *alk*⁻ *alcO*⁺)	NTG mutant of PpS126

[a] OCT and CAM-OCT are, respectively, the wild-type octane plasmid (5) and the fused plasmid (4) carrying the replication, transfer, and camphor utilization loci of the *P. putida* CAM plasmid (13) and the alkane utilization loci of the OCT plasmid (5). The genotypic symbols are explained in the legend to Table 9. We have designated the chromosomal locus for octanol utilization *alcA*; this has been previously referred to as *ocl* or *ool* (5).

Assay of whole-cell oxidizing activity. Warburg measurements were performed as described by Umbreit et al. (19). The flasks were incubated at 30.5 C and contained: 1 ml of bacteria suspended in PA salts plus 200 μg of chloramphenicol per ml to prevent enzyme synthesis during the measurements, 1 ml of substrate, and 0.2 ml of 20% KOH in the center well. Substrates were either dissolved in PA salts at the concentrations indicated or suspended in PA salts by sonication according to Robinson (14). High-vacuum silicone grease (Dow) was used to lubricate all joints because lanolin absorbs alkane vapors (14). For measurements with volatile substrates, we found it necessary to use a barometer containing 1 ml of substrate and 1 ml of PA salts in place of the bacterial suspension. All QO_2 values given are corrected for endogenous respiration.

A more convenient and sensitive method for determing whole-cell alkane oxidation is based on the evolution of $^{14}CO_2$ from labeled nonane. One milliliter of cell suspension was incubated for 60 min at 32 C with gentle agitation in a 50-ml Erlenmeyer flask containing 1 ml of a [1-^{14}C]nonane suspension in PA salts prepared by sonication. The flask was sealed with a serum vial stopper, and a plastic cup was suspended from the stopper. The cup contained a pleated piece of filter paper saturated with 20% KOH. After the incubation, the assay was terminated by opening the flask, removing the filter paper, and measuring the $^{14}CO_2$ evolved by counting the filter paper in a Packard Tri-Carb scintillation counter. Residual KOH solution in the cups was soaked up with a second piece of filter paper and counted together with the first. The quenching for all samples was virtually the same. The alkalinity of the sample causes considerable chemiluminescence of the scintillation fluid, and the vials must be kept dark and allowed to equilibrate for at least 1 h before counting. Activity is expressed as counts/min of $^{14}CO_2$ detected per milligram (dry weight) of cells. The background radioactivity due to nonane vapors adsorbing to the filter paper was measured by incubating a flask without bacteria; this was generally less than 100 counts/min and has been subtracted from all the values given below.

Preliminary experiments indicated that the radioassay is linear with respect to bacterial concentration, time, and substrate concentration in the range of 0.005 to 0.05%. The experiments presented below were all performed with a final concentration of 0.05% [1-^{14}C]nonane (5.63 μmol/flask) at a specific activity of 100 μCi of nonane per ml.

In all experiments dry weight of the cells was calculated from optical density measurements in a Klett colorimeter which had previously been calibrated.

Induction procedure. In most of the experiments reported here, cells were exposed to inducer during exponential growth in PA salts plus carbon source at 32 C on a rotary shaker with vigorous aeration. Inducing compounds of low solubility were dispensed in a small volume of PA salts by sonication before being added to the culture. At the appropriate time, cell samples were withdrawn, chloramphenicol (200 μg/ml) was added to prevent further protein synthesis, the samples were centrifuged at low speed, and

the pellet was suspended in PA salts plus chloramphenicol (200 μg/ml). In several of the experiments, the cells were suspended in PA salts lacking NH_4Cl.

For the experiments reported in Table 2, cells were grown overnight at 32 C as confluent lawns on PA agar with 0.4% glucose incorporated in the agar or alkanes were added in the vapor phase.

RESULTS

Inducibility of whole-cell alkane-oxidizing activity. Growth of *P. putida* PpG6 on purified *n*-alkanes results in the appearance of alkane-oxidizing activity which is not found in glucose-grown cells (Table 2). Growth on a single alkane substrate (octane) induces oxidizing activity for all five alkanes which support the growth of this strain (experiment 13). This indicates a single inducible enzyme or group of enzymes for the oxidation of all alkane growth substrates. This conclusion agrees with the genetic data of Nieder and Shapiro (10), which show that strain PpG6 has a single alkane hydroxylase activity. The fact that growth on or exposure to heptane, octane, or decane induces this activity (Table 2) further indicates that there is a single induction mechanism which responds to any one of the alkane growth substrates. Thus, it appears that any one of these alkanes is equivalent to any other both as inducer and as oxidation substrate.

Induction of alkane-oxidizing activity in growing cultures. Van Eyk and Bartels (22) had demonstrated that cultures of *Pseudomonas aeruginosa* 473 growing in enriched minimal salts-malonate medium could be induced to oxidize hexane by a number of alkane analogues. We confirmed their results for DCPM and dimethoxyethane with strain PpG6 (Fig. 1). In addition, we found that DCPK is an effective inducer of heptane-oxidizing activity (Fig. 1). Since malonate is a very poor carbon source for *P. putida* (doubling time of over 3 h at 32 C), we tested induction of alkane-oxidizing activity by DCPK in a number of different media. Figure 2 shows that pyruvate exerts little "catabolite repression" in PA salts medium and was used as the carbon source in the following experiments.

Nature of the induced activities. Assays of whole-cell oxidation measure the total activity of a large number of enzymatic reactions. To determine which of these reactions are induced in response to alkanes or DCPK, we performed the experiment presented in Table 3. Oxidizing activities for aliphatic aldehydes and fatty acids

TABLE 2. *Inducibility of alkane-oxidizing activity in P. putida PpG6*[a]

Expt	Growth substrate (inducer)	QO_2				
		Hexane	Heptane	Octane	Nonane	Decane
4	Glucose			5		
	Octane			172		
6	Glucose		0			
	Heptane		97			
13	Octane	131	104	146	138	92
15[b]	None					5
	Decane		156	132		48

[a] Cultures of strain PpG6 were grown on solid medium and assayed as described in Materials and Methods. Alkane substrates in the Warburg assay were present at a final concentration of 0.5%.

[b] In experiment 15, glucose-grown cultures were starved with aeration in PA salts for 4 h at 32 C with or without 0.4% decane before washing and assay.

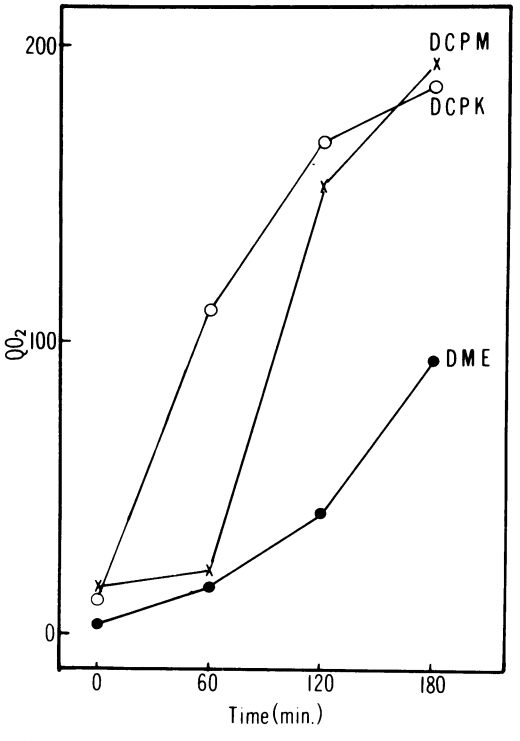

FIG. 1. *Induction of heptane-oxidizing activity in liquid culture. Cultures of P. putida PpG6 growing in PA salts plus 20 mM malonate and 0.01% yeast extract were induced by the addition of 5 mM DCPK* (○), *5 mM DCPM* (×), *or 50 mM dimethoxyethane* (*DME*, ●). *At the times indicated on the abscissa, samples were removed and assayed for heptane oxidation in a Warburg respirometer as described in Materials and Methods (final concentration of substate, 0.5%).*

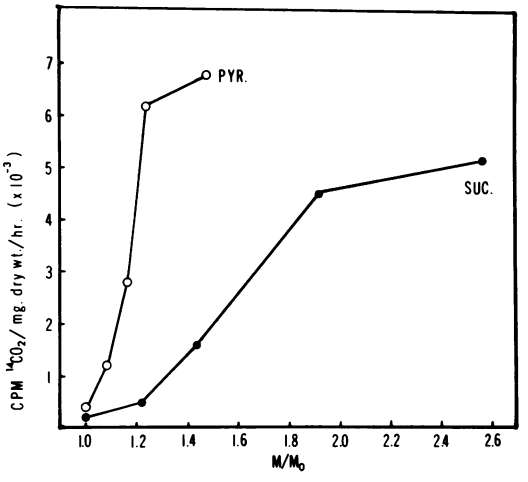

FIG. 2. *Effect of different carbon sources on the induction of alkane-oxidizing activity in P. putida PpG6. Exponentially growing cultures of PpG6 in PA salts plus 0.5% carbon source were induced with DCPK (1 mM). At 0, 15, 30, 60, and 90 min, samples were removed and assayed for nonane-oxidizing activity. Specific activity is plotted against the relative increase in cell mass as determined by optical density measurements. Symbols:* ○, *pyruvate;* ●, *succinate.*

TABLE 3. *Induction of oxidizing activity for intermediates in heptane oxidation*[a]

Inducer	[1-^{14}C]nonane oxidizing activity (counts/min of $^{14}CO_2$/mg [dry wt]/h)	QO_2		
		1-Heptanol	Heptanal	Heptanoic
None	287	62	202	214
Heptane (0.4%)	10,005	273	235	258
DCPK (1 mM)	13,445	215	167	163

[a] Exponentially growing cultures of strain PpG6 in PA salts-0.5% pyruvate medium were induced for 60 min, harvested, and assayed as described in Materials and Methods. All Warburg substrates were present at a final concentration of 0.05%. Since aliphatic alcohols, aldehydes, and fatty acids are toxic, low concentrations ($\leq$0.05%) must be used in Warburg experiments to obtain reliable results (14).

in strain PpG6 are clearly constitutive. This confirms previous observations using glucose-grown cells and a series of fatty acids from propanoic to nonanoic (M. Nieder, M. Toepfer, J. Leahy, and J. Shapiro, unpublished data). There is a significant basal level of primary alcohol-oxidizing activity, but induction by heptane or DCPK increases this activity approximately fourfold. Apparently, therefore, one of the induced activities is an alcohol dehydrogenase. Alkane-oxidizing activity is induced at least 20-fold by heptane and DCPK. Thus, alkane-hydroxylating activity is also inducible. Comparison of alkane- and alcohol-oxidizing activities in cultures of strain PpG6 and a derivative cured of the OCT plasmid (Table 4) shows that the inducible hydroxylase and dehydrogenase activities are plasmid coded.

Induction by alkane oxidation products. Table 5 shows the inducer activity of the different intermediates in the alkane to fatty acid pathway. Heptanol is an effective inducer, heptanal induces weakly, and heptanoic acid is not an inducer. The observation that heptanol induces suggested that alkane-oxidizing activity might be regulated by product induction. Various authors have observed a lag in induc-

TABLE 4. *Induction of heptanol- and nonane-oxidizing activity in OCT$^+$ and OCT$^-$* [a]

Strain	Inducer	QO_2 (heptanol)	[1-^{14}C]nonane oxidizing activity (counts/min of $^{14}CO_2$/mg [dry wt]/h)
PpG6 (OCT$^+$)	None	31	331
	DCPK (1 mM)	178	5,665
	Heptane	227	7,268
PpS4 (OCT$^-$)	None	32	123
	DCPK (1 mM)	31	186
	Heptane	37	162

[a] PpS4 (OCT$^-$) is a derivative of strain PpG6 that has been cured of the OCT plasmid by mitomycin C treatment. Exponentially growing cultures in minimal salts-0.5% pyruvate medium were induced for 60 min and assayed as described in Materials and Methods.

TABLE 5. *Induction of whole-cell alkane-oxidizing activity by oxidation pathway intermediates*[a]

Inducer	[1-^{14}C]nonane oxidizing activity (counts/min of $^{14}CO_2$/mg [dry wt]/h)
None	287
Heptane (0.4%)	7,044
1-Heptanol (5 mM)	4,351
Heptanal (5 mM)	1,995
Heptanoic acid (5 mM)	457

[a] Cultures of strain PpG6 were induced for 60 min in PA salts-0.5% pyruvate medium and assayed as described in Materials and Methods.

tion by alkane substrates (1, 22), and this lag could be explained as the time needed for basal hydroxylase activity to form sufficient primary alcohol for induction to occur. Accordingly, we determined the kinetics of induction by heptane and 1-heptanol (Fig. 3). Both compounds display the same 10-min lag. Thus, the observed lag cannot be due to the formation of an alcohol inducer. Further evidence that recognition of the primary alcohol inducer does not involve the hydroxylated end of the molecule comes from the experiment presented in Table 6,A. The presence of terminal or subterminal hydroxyl groups at both ends of a carbon chain effectively prevents induction. As the control experiment with DCPK plus hexanediol demonstrates (Table 6,B), the absence of activity after exposure to the diol cannot be due to irreversible inhibition of hydroxylase activity or cell toxicity. Similar results are obtained with hexanediol concentrations in the range of 1 to 20 mM. Thus, it appears that primary alcohols are effective inducers, because one end of the carbon chain mimics the unoxidized alkane molecule. The induction specificity of *P. putida* PpG6 is different from that of *P. aeruginosa* 473 because the latter is inducible by 1,6-hexanediol (20). Table 7 shows that there is a chain-length

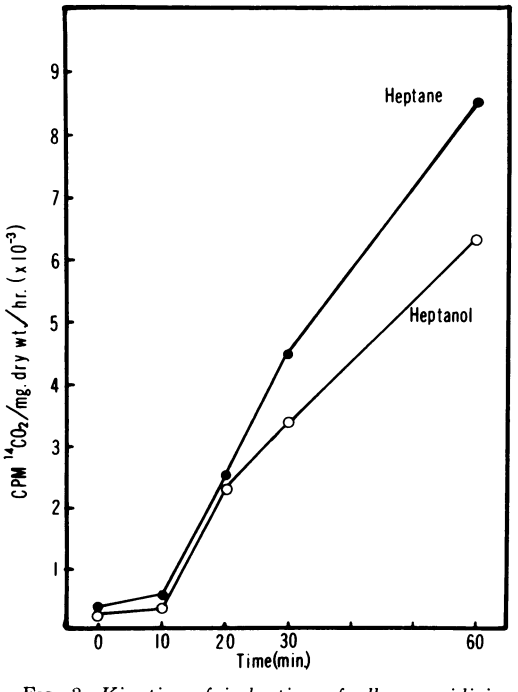

FIG. 3. *Kinetics of induction of alkane-oxidizing activity in P. putida PpG6. An exponentially growing culture of strain PpG6 in PA salts-0.5% pyruvate medium was divided and induced with 0.4% heptane (●) or 5 mM 1-heptanol (○). At the times indicated on the abscissa, samples were removed and assayed for nonane-oxidizing activity as described in Materials and Methods.*

TABLE 6. *Induction of alkane-oxidizing activity by six-carbon alcohols and diols*[a]

Inducer	[1-^{14}C]nonane oxidizing activity (counts/min of $^{14}CO_2$/mg [dry wt]/h)
A	
None	235
1-Hexanol (1 mM)	4,933
1,6-Hexanediol (5 mM)	541
2-Hexanol (3 mM)	4,892
2,5-Hexanediol (5 mM)	387
B	
1,6-Hexanediol (5 mM)	437
DCPK (1 mM)	5,855
1,6-Hexanediol (5 mM) + DCPK (1 mM)	6,093

[a] Cultures of strain PpG6 were induced for 60 min in PA salts-0.5% pyruvate medium and assayed as described in Materials and Methods.

TABLE 7. *Induction of alkane-oxidizing activity by various compounds*[a]

Compound	Inducer	[1-^{14}C]nonane oxidizing activity (counts/min of $^{14}CO_2$/mg [dry wt]/h)[b]
Primary alcohols	None	388
	1-Propanol (3 mM)	434 (2)
	1-Butanol (3 mM)	285 (2)
	1-Pentanol (3 mM)	4,737 (2)
	1-Dodecanol (1 mM)	159 (2)
Miscellaneous compounds	Diethoxyethane (10 mM)	4,335
	DCPK (1 mM)	9,022
	DCPM (5 mM)	7,067
	1,7-Octadiene (0.4%)	7,030
	2-Methylhexane (0.4%)	2,706
	3-Hexanol (3 mM)	8,162
	2-Heptanone (0.4%)	6,255
	2-Octanone (0.4%)	7,128
Alkanes that are not growth substrates	*n*-Undecane (0.4%)	243 (2)
	n-Dodecane (0.4%)	328 (2)

[a] Cultures of strain PpG6 were induced for 60 min in PA salts-0.5% pyruvate medium and assayed as described in Materials and Methods.

[b] The numbers in parentheses indicate the number of independent determinations averaged to give the values presented here.

specificity to induction by primary alcohols; propanol, butanol, and 1-dodecanol do not act as inducers.

Induction by other compounds. In addition to DCPK, DCPM, dimethoxyethane, and primary alcohols, several compounds are effective inducers of alkane-oxidizing activity (Table 7, miscellaneous compounds). Many of these inducers do not serve as substrates either for growth or respiration (Table 8). (The low QO_2 values observed for DCPK and 2-hexanol are probably not significant.) Many of these inducers, moreover, are toxic to *P. putida* cells above a certain concentration, and it is sometimes necessary to determine the optimum inducing concentration. Use of too much inducer (e.g., 10 mM 1-hexanol, 50 mM DCPM, or 50 mM diethoxyethane) as well as too little (e.g., 1 mM DCPM or 5 mM diethoxyethane) can lead to false-negative results (A. Grund, unpublished data). The data in Table 7 show that two *n*-alkanes which do not serve as growth substrates, undecane and dodecane (10), are also not inducers of alkane-oxidizing activity.

Induction requires transcription. Van Eyk and Bartels (22) have shown that exposure to hexane induces oxidizing activity detectable in crude-cell extracts of *P. aeruginosa* 473, and we have found that either heptane or DCPK induces alkane hydroxylase activity detectable in cell-free extracts of strain PpG6 (3). Thus, induction results in enzyme synthesis and is not due to a permeability change. Figure 4 shows that rifampin blocks the synthesis of alkane-oxidizing activity. Hence, induction requires the synthesis of ribonucleic acid.

Isolation of alkane oxidation pathway mutants. Our physiological data demonstrated the existence of inducible plasmid-coded oxidizing activities for alkanes and primary alcohols and constitutive chromosomally coded oxidizing activities for primary alcohols, aldehydes, and fatty acids (Tables 3 and 4). To confirm these conclusions, we isolated mutants unable to grow on alkanes or their oxidation products and then determined the step at which they were blocked by growth tests. The results for a number of different strains are summarized in

TABLE 8. *Alkane analogues as substrates for growth and respiration*[a]

Compound	Ability to support growth of *P. putida* PpG6	Ability to serve as substrate for respiration by *P. putida* PpG6 cultures		
		Concn	QO_2	Inducer
DCPK	–	1 mM	0	None
		5 mM	0	None
		1 mM	4.7	DCPK (1 mM)
		5 mM	0	DCPK (1 mM)
		10 mM	1.6	DCPK (1 mM)
DCPM	–	5 mM	0	DCPM (5 mM)
Diethoxyethane	–	10 mM	33	Diethoxyethane (10 mM)
2-Methylhexane	–	0.4%	0	None
		0.4%	0	DCPK (1 mM)
2-Hexanol	–	3 mM	1	None
		3 mM	4	DCPK (1 mM)
3-Hexanol	–	3 mM	11	None
		3 mM	0	DCPK (1 mM)
1,7-Octadiene	+	0.4%	4	None
		0.4%	36	DCPK (1 mM)
2-Heptanone	–		NT	
2-Octanone	–		NT	

[a] Growth tests and Warburg assays were performed as described in Materials and Methods. Induced cultures were induced for 60 min in PA salts-0.5% pyruvate medium; the inducer used is indicated in the last column. Substrates for the Warburg assays were added at the concentration indicated in the third column. NT, Not tested.

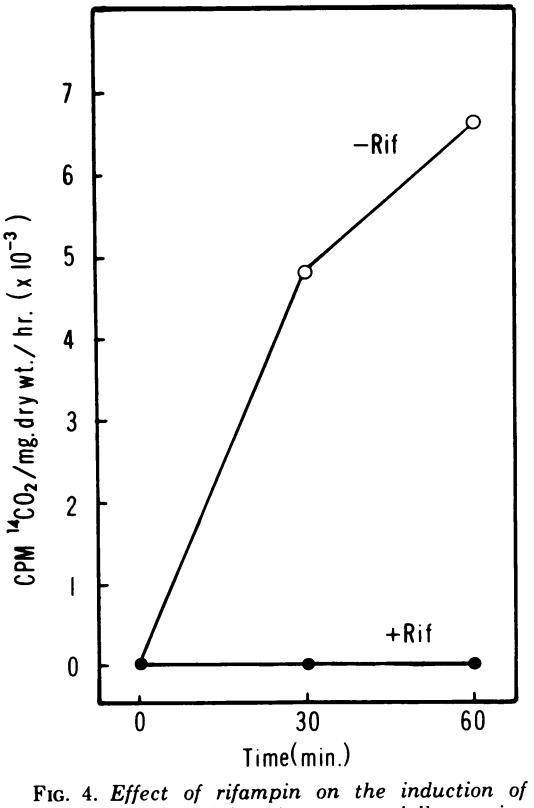

FIG. 4. *Effect of rifampin on the induction of alkane-oxidizing activity. An exponentially growing culture of strain PpG6 in PA salts-0.5% pyruvate medium was induced with 1 mM DCPK* (○). *At 0 min* (●) *200 μg of rifampin per ml was added to a portion of the culture and incubation was continued. At the times indicated on the abscissa, samples were removed and assayed for nonane-oxidizing activity.*

Table 9. These data lead to the following conclusions. (i) The chromosome codes for at least two alcohol dehydrogenase activities; mutants of strain PpG1 with no plasmid selected for inability to grow on octanol or nonanol can still grow on shorter-chain alcohols. (ii) The chromosome codes for two aldehyde dehydrogenase activities; mutants of strain PpG1 or PpS70 which cannot grow on nonanal are negative only for primary alcohols of seven or more carbon atoms. (iii) The chromosome codes for at least one activity common to the oxidation of all straight-chain fatty acids because *oic*$^-$ mutants are unable to grow on all the alcohols tested. (iv) The plasmid codes for a redundant alcohol dehydrogenase activity because no alcohol-negative mutants were found among mutants selected from strain PpG6 for inability to grow on octane or nonane, but such mutants are readily isolated from strain PpS70, which carries a chromosomal mutation blocking growth on octanol and nonanol. We have confirmed that at least one of these mutants is hydroxylase positive and dehydrogenase negative by crossing the mutant plasmid to strain PpG1 (*alcA*$^+$) and obtaining alkane-positive exconjugants, whereas a control cross to strains PpS81 (*alcA*$^-$) yielded no such exconjugants. (v) The plasmid does not code for an aldehyde dehydrogenase activity because aldehyde-negative mutants are found in strain PpS70, which has both the OCT plasmid and the chromosomal loci for aldehyde utilization. This conclusion was confirmed by crossing the OCT$^+$ *met*$^-$ strain PpS5 with an aldehyde-negative OCT$^-$ mutant and selecting nonanal-positive prototrophic recombinants; less than 2% of the recombinants had inherited the OCT plasmid, indicating that inheritance of the plasmid cannot correct the chromosomal block in aldehyde utilization. (In contrast, the same cross with an *alcA*$^-$ recipient strain yields more than 50% OCT$^+$ recombinants when they

TABLE 9. *Isolation of P. putida mutants affected in the metabolism of alkanes and their oxidation products*[a]

Parent	PpG1 (WT, OCT$^-$)					PpG6 (WT, OCT$^+$)			PpS70 (*alcA*$^-$ *str*, OCT$^+$)				
Selected phenotype	Octanol-negative					Octane- or nonane-negative			Heptane-negative				
Mutant types	Parent	*alcA*$^-$	*aldA*$^-$	*oic*$^-$	*ace*$^-$	parent	*alk*$^-$	*ace*$^-$	parent	*alk*$^-$	*alcO*$^-$	*aldA*	*oic*$^-$
No. of mutants isolated in each class		5	2	1	1		6	1		6	7	1	1
Growth on alkanes													
Hexane						+	−	−					
Heptane						+	−	+	+	−	−	−	−
Octane						+	−	−	+		−		
Nonane						+	−	+					
Decane						+	−	−					
Growth on primary alcohols													
1-Dodecanol	+	−				+			−		−		
1-Nonanol	+	−	−	−	+	+	+	+	+	+	−	−	−
1-Octanol	+	−	−	−	−	+	+	−	+	+	−	−	−
1-Heptanol	+	?	−	−	+	+			+		−	−	−
1-Hexanol	+	+	+	−	−	+			+		+	+	−
1-Pentanol	+	+	+	−	+	+			+		+	+	−
1-Butanol	+	+	+	−	−	+			+		+	+	−
1-Propanol	+	+	+	−	+	+			+		+	+	−
Nonanal	+	+	−	−	+	+			+	+	+	−	−
Nonanoic acid	+	+	+	−	+	+	+	+	+	+	+	+	−
Octanoic acid	+	+	+	−	−	+	+	−	+	+	+	+	−
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] Mutants were isolated and tested as described previously (10). The phenotypic symbols employed are: *alc*, growth on primary alcohols; *ald*, growth on aliphatic aldehydes; *oic*, growth on straight-chain fatty acids; *ace*, growth on acetate; and *alk*, growth on alkanes. The *alc*A locus is the chromosomal locus which codes for growth on octanol, nonanol, and dodecanol; this has previously been called *oc1* or *oo1* (5). The *alc*O locus is the plasmid locus which permits growth on octanol and nonanol in the presence of an *alc*A$^-$ mutation. Mutants negative on nonanal and primary alcohols of seven or more carbon atoms are designated *ald*A$^-$ because we presume that there is at least one other *ald* locus which codes for growth on shorter aldehydes. The data for mutants from strain PpG6 is taken from Nieder and Shapiro (10). WT, Wild type.

are selected for growth on nonanol.) (vi) The specificity of the plasmid-coded alcohol dehydrogenase appears to differ from that of the corresponding chromosomal enzyme. Strains PpG1 and PpG6 with the chromosomal enzyme are able to grow on 1-dodecanol, whereas strain PpS70, which has only the plasmid enzyme, is negative on 1-dodecanol. Strains carrying the fused CAM-OCT plasmid yield the same mutant types after nitrosoguanidine mutagenesis.

Role of induction in determining growth specificity. *P. putida* strains carrying the OCT or fused CAM-OCT plasmid cannot grow on undecane or dodecane but can grow on dodecanol if they carry the *alcA*$^+$ allele on the chromsome (10; Table 9). Thus, the growth-negative phenotype on these alkanes is due to lack of hydroxylating activity (10). This can be explained either by the substrate specificity of the hydroxylase enzyme or by lack of induction. Two lines of evidence suggested that lack of induction is the correct explanation. First, McKenna and Coon (9) have shown that the OCT-coded alkane hydroxylase has 47% of maximal activity in vitro with dodecane as substrate. Although in vitro and in vivo substrate specificities may differ, this level of activity should support growth if present in the cells. Second, undecane and dodecane do not act as inducers of whole-cell alkane-oxidizing activity (Table 7). To show that lack of induction is indeed the reason that OCT$^+$ and CAM-OCT$^+$ strains cannot grow on undecane, we performed the experiment presented in Table 10. Addition of DCPK to the medium permits slow growth on undecane by OCT$^+$ and CAM-OCT$^+$ strains. (We had previously shown that strains carrying the fused plasmid are heptane and DCPK inducible for alkane-oxidizing activity.) The same experiment performed with dodecane suggests that OCT$^+$ and CAM-OCT$^+$ strains can grow very slowly on this alkane in the presence of DCPK; however, the growth is very faint after 96 h of incubation, and the plates are difficult to score reliably.

A similar explanation could account for the inability of *alcA*$^-$ (OCT$^+$) and *alcA*$^-$ (CAM-OCT$^+$) strains to grow on 1-dodecanol (Table 9), since this compound is not an inducer of whole-cell alkane-oxidizing activity (Table 7). The results in Table 10 show that induction of the plasmid alcohol dehydrogenase activity by DCPK does permit growth on dodecanol.

Induction of plasmid alcohol-oxidizing activity in hydroxylase-negative mutants. The results in Fig. 3 and Table 6 do not provide evidence for the product induction model and indicate that inducer recognition is not specific for the hydroxyl group of aliphatic alcohols. Our results remain consistent with any of three models for the induction of the two plasmid-coded activities: coordinate induction by the initial substrate (alkane), coordinate induction by the product (primary alcohol), or sequential induction. One prediction unique to the first model is that an alkane inducer should induce plasmid alcohol-oxidizing activity in the absence of hydroxylation to the primary alcohol. The results in Table 10 suggested a method of testing this prediction. This is to determine whether alkane induction would permit growth on 1-dodecanol by *alk*$^-$ mutants isolated from *alcA*$^-$ strains lacking the chromosomal alcohol dehydrogenase activity. Accordingly, we performed the growth tests summarized in Table 11. The heptane used in this experiment was purified by passage through a silicic acid column to eliminate any possible contamination by alkane oxidation products. The results indicate that purified heptane can induce the plasmid alcohol-oxidizing activity in the absence of hydroxylation and therefore support the model of coordinate induction by unoxidized alkane substrate. A similar result for induction of alkane hydroxylase proteins is reported in the accompanying paper (3).

TABLE 10. *Effect of DCPK on the substrate range of strains carrying the CAM-OCT plasmid*[a]

Strain	Growth on PA agar plus				
	DCPK[b]	1-Dodecanol		*n*-Undecane	
		None[c]	DCPK	None	DCPK
PpG6 *alcA*$^+$ (OCT)	–	++	NT	–	+
PpS124 *alcA*$^+$ (CAM-OCT)	–	++	++	–	+
PpS125 *alcA*$^-$ (CAM-OCT)	–	–	++	–	+
PpS126 *alcA*$^-$ (CAM-OCT)	–	–	++	–	+

[a] The strains were streaked on PA agar and PA agar containing 5×10^{-4} M DCPK and exposed to substrate vapors for 48 h (dodecanol) or 96 h (undecane) at 32 C in sealed tins. Growth was scored by visual inspection: ++, normal dense bacterial growth yielding isolated colonies 1 to 2 mm in diameter; +, clearly visible growth with microcolonies. Genotypic symbols are explained in the footnote to Table 9. NT, Not tested.

[b] Substrate.

[c] Inducer.

TABLE 11. *Phenotypic test for induction of plasmid-coded alcohol dehydrogenase activity in hydroxylase-negative mutants*[a]

Strain	Growth on PA agar plus				
	Heptane	1-Nonanol	1-Dodecanol	1-Dodecanol + DCPK	1-Dodecanol + heptane
PpS84 *alcA*$^-$(OCT*alk*$^-$*alcO*$^+$)	−	+	−	+	+
PpS88 *alcA*$^-$(OCT*alk*$^-$*alcO*$^+$)	−	+	−	+	+
PpS181 *alcA*$^-$(CAM-OCT*alk*$^-$*alcO*$^+$	−	+	−	+	+
PpS191 *alcA*$^-$(CAM-OCT*alk*$^-$*alcO*$^+$)	−	+	−	+	+

[a] Growth tests were performed as described in the footnote to Table 10 (+, normal growth). Silicic acid-purified heptane was added in the vapor phase. Control plates showed that *alk*$^+$ strains would give positive growth on heptane agar under these conditions. All four mutants were independently induced by nitrosoguanidine treatment. Genotypic symbols are explained in the footnote to Table 9.

DISCUSSION

Our conclusions about the plasmid and chromosomal loci involved in the oxidation of alkanes and their physiological derivatives by *P. putida* strains are presented schematically in Fig. 5. This picture is derived from the physiological data in Tables 3 and 4, the genetic data in Table 9, and the results of Nieder and Shapiro (10). In the case of the *alk* locus, we have indicated more than one cistron based on biochemical data (6, 8, 12). The existence of an OCT plasmid-coded primary aliphatic alcohol dehydrogenase was already known from the work of Chakrabarty et al. (5).

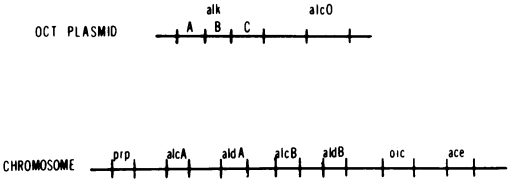

FIG. 5. *P. putida loci involved in alkane oxidation. Most of the genotypic symbols are explained in the legend to Table 9; prp, propionate utilization (10). Except for the assignment of a given locus to the chromosome or the OCT plasmid, the locations of the different loci are arbitrary.*

One interesting aspect of this picture is that there are several enzyme redundancies for similar or identical biochemical reactions. A parallel situation exists in *P. aeruginosa* strains for aliphatic alcohol dehydrogenase activities (15, 21) and in at least one *P. aeruginosa* strain for alkane hydroxylase activity (10). This means that biochemical analysis of alkane oxidation and its regulation will have to distinguish between the different enzymatic activities. For example, strain PpG6 grown on alkanes contains a nicotinamide adenine dinucleotide-linked octanol dehydrogenase activity that can be measured in crude extracts, but this same activity is present in an *alcA*$^-$ strain without an OCT plasmid (Shapiro and Rhoades, unpublished data). Hence, it is almost certainly not the activity responsible for dehydrogenating octanol in vivo.

Another interesting feature is that the OCT plasmid apparently codes only for the first two steps in alkane oxidation and not the entire alkane to fatty acid pathway. Since alkane hydroxylation by itself would be sufficient to bring these substrates into the range of chromosomally determined cellular metabolism, it remains a mystery why the plasmid evolved with its own inducible primary aliphatic alcohol dehydrogenase.

As the results in Table 10 indicate, the specificity of the induction mechanism for alkane hydroxylase and alcohol dehydrogenase activities can determine the range of growth substrates for *P. putida* strains carrying the OCT plasmid. For both the plasmid hydroxylating and alcohol dehydrogenating activities, the range of substrates is more extended than the range of inducers. It would not be surprising if the substrate ranges of other alkane-oxidizing bacteria are similarly limited by inducer specificity. The existence of noninducing substrates means that it will be possible to isolate constitutive mutants.

If we assume that inducer molecules are not metabolized before acting, then the data presented above allow us to reach some tentative conclusions about molecular requirements for inducer recognition. A straight carbon or carbon-oxygen chain of six to ten atoms in length can give inducer activity (Tables 2, 3, and 7). One end of the chain must be without terminal or subterminal hydroxyl groups (Table 6). A

ketone, hydroxyl, or methyl group on the subterminal carbon does not prevent induction, although the latter reduces effectiveness (Tables 6 and 7). An aldehyde group also reduces inducer activity, and a carboxyl group eliminates it (Table 5). This may be related to the high consitutitive level of aldehyde and fatty acid oxidation (Table 3). From Table 7 it is clear that the length of straight-chain molecules can determine whether or not they act as inducers. In other words, the inducer recognition site can somehow measure the length of the carbon chain. This suggests that the recognition molecule contains a "pocket" into which both ends of the inducer molecule fit. DCPK and DCPM may act as inducers because they resemble alkane chains folded inside this pocket.

Our assumption that inducer molecules are not metabolized before acting remains to be rigorously demonstrated. It is based on results which show that some inducers are not growth substrates and which indicate that some are also not respiration substrates (Table 8). The Warburg experiments are rather imprecise, but even if our interpretation is correct the results do not preclude metabolic transformations which stop before the inducer molecule can enter carbon- or energy-yielding pathways. The results in Table 11 indicate that alkanes can induce the plasmid alcohol-oxidizing activity in the absence of hydroxylation. Trivial explanations for these results (such as a residual level of hydroxylation insufficent for growth) have not been excluded, but the results nonetheless suggest that both plasmid activities are under coordinate control and that the alkane is the physiological inducer. This is consistent with the results in Fig. 3 and Table 6, which show that induction is not alcohol specific, and with the fact that a molecule like DCPK induces both plasmid activities. Induction requires transcription of ribonucleic acid (Fig. 4) and presumably involves one or more regulatory proteins. Conclusive evidence for coordinate control and the elucidation of whether the postulated regulatory proteins act in a negative or positive fashion will require the isolation of constitutive and noninducible mutants. We are currently searching for these mutants.

ACKNOWLEDGMENTS

We wish to thank Wolfgang Epstein, David Rhoades, and Spencer Benson for helpful discussions and Lucia Rothman-Denes for her many valuable suggestions and for reading the manuscript.

Alan Grund was the recipient of a summer research stipend from the Collegiate Division of Biological Sciences of the University of Chicago. Michael Fennewald is the recipient of a Public Health Service predoctoral traineeship. This work was begun during an undergraduate course supported by the Collegiate Division of Biological Sciences of the University of Chicago and has been supported in part by grants from the Louis Block Fund of the University of Chicago and the National Science Foundation.

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