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INVESTIGATIONS INTO THE GENETIC BASIS OF THE BIODEGRADATION OF PHENOLIC WASTES IN SELECTED STRAINS OF PSEUDOMONAS

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Two species of bacteria, Pseudomonas aeruginosa and Pseudomonas putida have been studied to determine the induction controls governing the membrane-bound hydoxylases and oxygenases responsible for cresol degradation. Interrupted mating, oxygen probe and spectrophotometric enzyme studies suggest a late chromosomal location for information controlling the expression of the 2,3 oxygenase for 4 methyl and 3 methyl catechol. P. aeruginosa JPT3-4 plasmid DNA transforms P. putida AC137 TOL+ to cresol competency at low frequency. Such transformation is apparently linked to a plasmid incompatible with TOL. The exact type of information transferred is not yet known. It is hoped that these studies on genetic mechanisms will lead to a strain of bacteria with greater cresol degrading abilities.

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FINAL PROGRESS REPORT ON AFOSR GRANT 76-2875

Investigations into the Genetic Basis of the Biodegradation of Phenolic Wastes in Selected Strains of *Pseudomonas*

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San Antonio, Texas

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1979

Introduction.

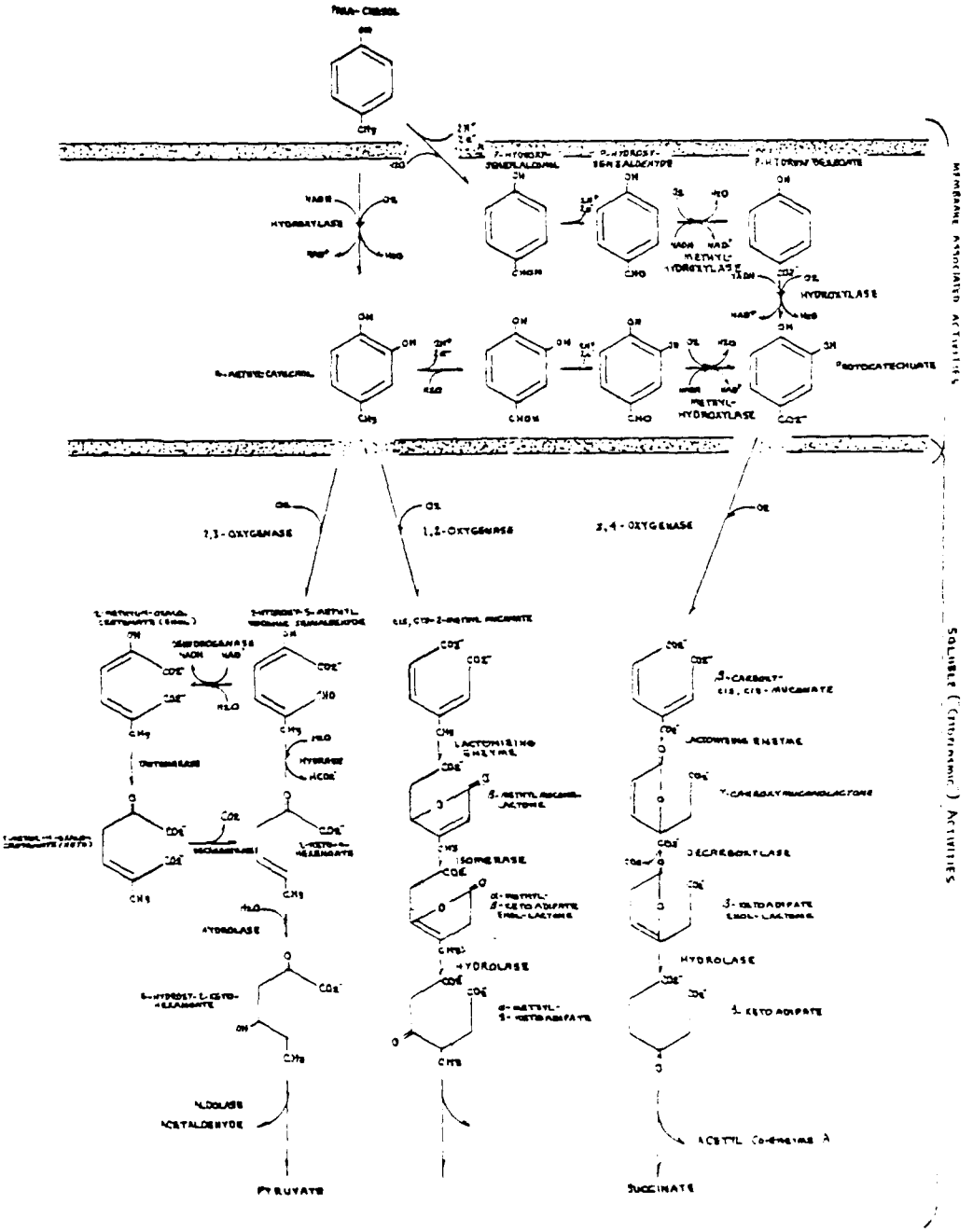
The past decade has seen a literal explosion in the application of microbial metabolism to waste management problems, and a parallel growth of interest in the genetic mechanisms which underlie such capacities. This laboratory, under the auspices of the AFOSR and, for a time, the AF Systems Command, was responsible for engineering an artificial ecosystem of organisms capable of handling concentrated phenolic wastes at extremely high efficiencies (1, 2, 3). Simultaneously, studies were also undertaken to develop an understanding of the genetic mechanisms characteristic of organisms adapted for use in phenolics biodegradation tasks.

Investigations by several other laboratories in the field of microbial metabolism have revealed an almost staggering diversity of pathways possessed by the world's microbial community. Among the most versatile has proven to be the group of bacteria known as the Pseudomonads (4). Although these organisms are the best studied soil microbes, we have identified a Corynebacterium species demonstrating similar metabolic capacities, and have included it in the scope of our studies (3).

Specifically with regard to the metabolism of aromatic carbon compounds, workers have identified two major modes of ring cleavage: intra-diol, or "ortho" ring-fission; and extra-diol, or "meta" ring-fission (see Figure 1), with many variations involving specific precursors and fission sites.

FIGURE 1a

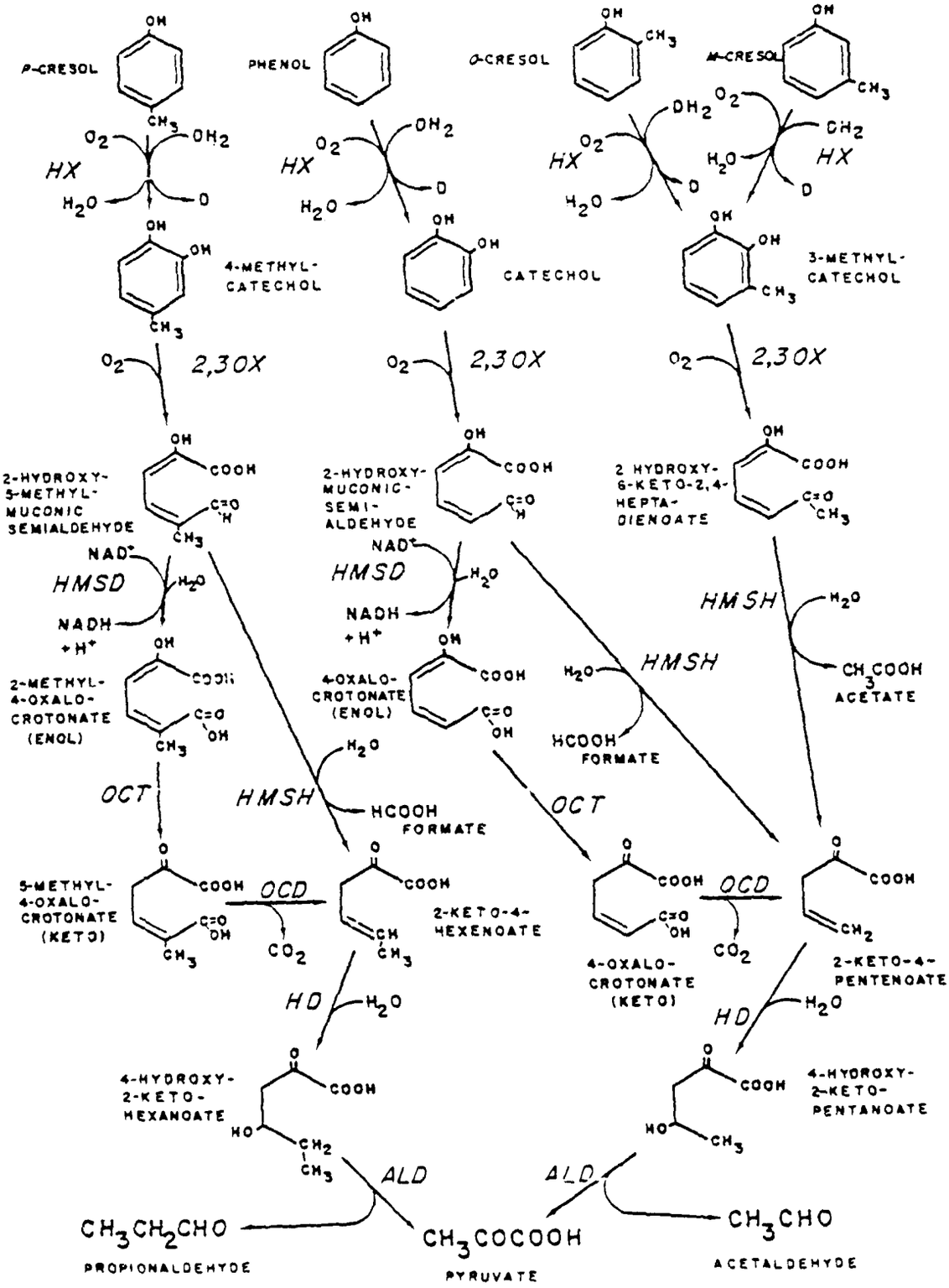
COMBINED DRAWING OF P-CRESOL RING-FISSION PATHWAYS KNOWN OR SUSPECTED IN PSEUDOMONAS JPT3-4 and JPT8 and CORYNEBACTERIUM J20



Para-cresol methylhydroxylases are active when separated from cell membrane. Ring hydroxylases function only when membrane-bound. The 4MCAT→PROCAT is a hypothetical sequence which may exist in JPT3-4, in addition to the other ortho- and meta-fission pathways. JPT8 appears to possess only 1,2- and 3,4-oxygenases, and may or may not carry genes coding for lower meta-pathway enzymes. JPT8 definitely possesses meta-cresol hydroxylase, inducible by para-cresol.

FIGURE 1b

META-RING-FISSION OF PHENOLICS IN PSEUDOMONAS J1 and JPT3-4



Typical waste degrading organisms demonstrate complex induction and loss-of-induction (metabolic shutdown) responses when presented phenolic substrates in combination with other carbon sources. Selection pressures on wild-type as well as engineered strains under the abnormal situations existing in phenolics degradation facilities both favor new gene constellations, and promulgate certain changes introduced during any prior laboratory manipulations, resulting in unique induction control systems. An understanding of what these changes are, and how to deliberately arrive at them, and maintain them, is one application for the type of basic research reported herein. Our own studies have indicated that our organisms--adapted to high concentrations (1000+ ppm) of the cresols, phenol and benzoate--possess at least two specific ring-fission pathways (5), but may differ from the wild-type in induction control (3), membrane component recipe, and in the cellular location of critical structural or functional genes.

Curing studies have indicated that, in Pseudomonas aeruginosa J1, at least some of the genes involved with the meta-cleavage pathway for the three cresol isomers are easily lost and may be carried on a plasmid. (Para-cresol utilization continues via a co-induced ortho-fission pathway.) Other investigators have identified plasmids involved with aromatic hydrocarbon degradation:

notably, Williams' and Worsey's TOL family of plasmids coding for meta-cleavage metabolism of toluene and xylene to pyruvate (6, 7); the Chakrabarty group's SAL (salicylate) and CAM (camphor) dissimilation plasmids (8, 9); and most recently, the ortho-cleavage-coding para-cresol degradation plasmid of Hewetson, Dunn and Dunn (10). We have examined strains of both Pseudomonas aeruginosa AC140 and P. putida AC137 bearing TOL plasmids, and found that they may, but do not necessarily, code for degradation of the cresols or phenol (see substrate utilization column in Table 1).

In P. aeruginosa JPT3-4, specificity for a cresol isomer or phenol seems to be shown in at least two steps: 1) at the level of initial transfer into the cell--what are called permease specificities; and 2) at the level of hydroxylation of the ring--a process that also appears to be associated with a membrane-bound enzyme. In some cases, it also appears that the catechol oxygenases of this organism show rather more specificity than was expected based on the work of other investigators. Thus it is possible that different enzymes, altered membrane proteins, and/or new structural genes are contributing to these strains' marked competency on phenolic substrates. Studies aimed at elucidating the genetic sites of these effects are currently underway. The results of investigations conducted from July 1978 to July 1979 are described in this report.

Bacterial Strains.

The primary cresol-degrading strains used in these studies were original isolates, or derivatives of isolates, obtained from the Kelly AFB-ALC depaint facility, San Antonio, Texas. Original strains J1, J2, J3, and J4 proved to be female, prototrophic fluorescent Pseudomonas aeruginosa; strains J20 and J24 were identified as Corynebacterium spp. (probably equi) (3). A number of cresol nonutilizing strains were obtained from other laboratories for use as marked recipients and as sources of known plasmids: AC137 and AC140 from A.M. Chakrabarty at General Electric's Physical Chemistry Laboratory; and numerous Pseudomonas aeruginosa and putida strains from Dr. James Walker, University of Texas at Austin, Department of Microbiology. Table 1 condenses strains and their characteristics.

Derived strains, such as JPT3, JPT3-4, and JPT8, were assembled in longterm matings of J1--primary subject of earlier studies at this laboratory--and PTO13, an auxotrophic (trp 6) mutant P. aeruginosa unable to degrade phenolics but possessing an FP2 sex factor plasmid. The male recombinant JPT3-4 was selected for playing the donor role in various mating experiments due to its metabolic similarity to the original J1, and to its neomycin antibiotic sensitivity (Nm^S).

Pseudomonas aeruginosa AC140 harbors the TOL plasmid, an extrachromosomal element known to code for a complete sequence of enzymes for the meta-fission pathway of toluene and xylene; the strain is marked by an amidase deletion (*ami*$^-$). *P. putida* AC137, a methionine-requiring (*met*$^-$) auxotroph also possessing a TOL-type plasmid, was also obtained for testing plasmid compatibilities, induction behaviour, and certain other parameters. Auxotrophic strains PAS102, PAS649, PAC5, Pps589, PAO 222, and PAO 225 were tested for usefulness as recipients in both interrupted mating and DNA transformation experiments to aid in genetic mapping of cresol metabolic (*cre*) loci.

Neomycin resistant (Nm^r) strains of potential recipients were obtained by longterm isolation on Luria/neomycin agar containing 400 ug/ml neomycin sulphate. Streptomycin resistant stocks (Str^r) were obtained in a similiar fashion, using streptomycin hydrochloride concentrations as high as 1000 ug/ml.

Strains were maintained on a number of media, in several stock modes. Longterm storage was effected on Luria and tryptic soy agar slants under sterile mineral oil at -40°C. Working auxotroph stocks were maintained on Luria slants, or on acetate or succinate minimal media agar slants supplemented with required amino acids, at room temperature. Working cresol-degrading stocks were maintained on Luria, or glucose or succinate minimal

media slants; and induced strains were stored on mineral salts agar supplemented with appropriate phenolic single carbon source, all at room temperature. Working antibiotic resistant strains were maintained on appropriate media with 400 ug/ml neomycin sulphate or 1000 ug/ml streptomycin hydrochloride, at room temperature. All room temperature stocks were transferred at two week intervals.

The phenotypic characteristics of most stock strains are shown in Table 1 .

TABLE 1

BACTERIAL STRAINS

STRAIN	GENUS	SPECIES	SOURCES/REFERENCES	GROWTH SUBSTRATES	MARKERS	PLASMIDS
J1,2,3,4	Pseudomonas	aeruginosa	KAFB-ALC, Bldg 375	PMOØBzTol$^-$	None	
J4-8	"	"	Mytomycin C, J1 (11)	P$^-$	None	
JPT3	"	"	J1 x PTO13	PMOØBzTol$^-$	None	FP2 variant
JPT3-4	"	"	Nm^s JPT3	PMOØBzTol$^-$	Nm^s	FP2 variant
JPT8	"	"	J1 x PTO13	PBz$^-$	None	
JPT8N	"	"	Nm^s JPT8	PBz$^-$	Nm^s	
AC137	Pseudomonas	putida	Chakrabarty	BTol$^+$	met$^-$	TOL-type
AC140	Pseudomonas	aeruginosa	Chakrabarty	Tol$^-$	ami^{del}	TOL-type
PAO222	"	"	Moody		his$^-$trp6$^-$met28$^-$ pro82$^-$ lys12$^-$ ilv226$^-$ StrA	
PTO13	"	"	Moody		trp6$^-$	FP2
PAC5	"	"	Meyers & Walker		his501$^-$	
PAC5SRN	"	"	Str^r & Nm^r PAC5		his501$^-$Str^{r}Nm^r	
PAS102	"	"	Meyers & Walker		thr102$^-$	
PAS102N	"	"	Nm^r PAS102		thr102$^-$Nm^r	
PAS649	"	"	Meyers & Walker		leu8$^-$	
PAS649N	"	"	Nm^r PAS649		leu8$^-$ Nm^r	
Pps589	Pseudomonas	putida	Meyers & Walker	PMBz$^-$	trp338$^-$ met589$^-$ alcA 437	
Pps589N	"	"	Nm^r Pps589	PMBz$^-$	trp338$^-$ met589$^-$ alcA 437 Nm^r	
J20	Coryne-bacterium	spp.	KAFB Bldg. 375	PMOØBzTol$^-$		

Oxygen Electrode Studies.

There are a number of enzymatic steps in the various ring-fissioning pathways (Figure 1) which require molecular oxygen. As a result, the activity of these enzymes can be assayed by feeding an induced, starved culture single, purified intermediates in the suspected pathways, then tracking the uptake of oxygen from the sealed test vessel using an oxygen-specific electrode. Comparison of these data with identical studies done on sonically disrupted cell preparations can aid in distinguishing permeability phenomena from actual pathway enzyme induction phenomena. Coupled with spectrophotometric enzyme assays, such studies can give fine scale information on induction mechanisms, and somewhat more ambivalent information on the presence or absence of specific genetic machinery.

Strain J1, subject of initial enzyme studies, revealed a meta-fission pathway inducible by all three isomers of cresol, with indications of either a second pathway induced by para-cresol, or a para-cresol-specific permease inducible by all three cresol isomers, coupled with a meta- and ortho-cresol specific permease inducible only by meta- and ortho-cresol (12).

Strain JPT3-4, obtained from a mate between J1 and PTO13 and selected for metabolic similarity to J1 coupled with possession of the FP2 sex factor, has since become the

chief target of donor-oriented characterization studies. As Table 2 indicates, its behavior on inducing substrates mimics that of J1. The more thorough current studies have shown that, indeed, two pathways are inducible by the cresol isomers: one, a classic meta-fission yielding yellow-colored intermediates; the other either an odd meta-cleavage variant, or an ortho pathway. Both pathways act on 4-methyl-catechol (4MCAT), yielding different subsequent ring-fission products. The meta-cleavage product is 2-hydroxy-5-methyl-muconic semialdehyde (HMMS); the other pathway's ring-fission product has not yet been positively identified, but spectrophotometric scans demonstrate a peak at 257 nm, similar to absorption spectra for the cis,cis-muconic acids produced in ortho fission (260 nm for catechol fission).

In the case of para-cresol-induced JPT3-4 and JPT8, 4MCAT is rapidly oxidized, but there is no sign of yellow ring-fission products. Absence of color and strong 4MCAT utilization are also associated with a marked protocatechuate (PROCAT) reaction. Apparently either the PROCAT oxygenase is co-induced with that for 4MCAT's fission, or the two enzymes are the same, or the PROCAT is an intermediate in the degradation of 4MCAT. (Conversion of the methyl group to carboxylate is enzymatically feasible.) These possibilities have not yet been resolved, and may prove to be beyond the scope of this study.

TABLE 2.

INDUCTION BEHAVIOR

		Test Substrate											
		Pre-hydroxylation				Post-hydroxylation							
	Inducing Substrate(s)	PCr	MCr	Ø	Bz	4-M-Cat		3-M-Cat		Cat		Pro-Cat	
Strain JPT3-4	PCr	++	w	-	-	+	y	+w		+w	yf	++	
	MCr	+	+	-	-	++	ys	+		+	yf	-	
	Bz	+	+	-	++	++	yf	+		++		+w	
	PCr+MCr	++	+	-	-	+++	ys	++		++	y	+	
	PCr+Bz	+	+	+w	++	++	ys	++	yf	++	y	++	
	PCr+Ac	++	++	NT	+w	++		+		+		+w	
	MCr+Bz	+	+	+	-	++	ys	+		+	yf	-	
	MCr+Ac	++	+	NT	-	++	y	++		+		-	
	Bz+Ac	++	+	-	++	+	ys	+		+		-	
	Ø+Bz	+	+	+	+	++	ys	++	yf	++	yf	-	
	Ø+Ac	++	+	++	+w	+++	ys	++		++	ys	-	
Strain JPT8N	PCr	++	+	-	-	+		-		-		++	
	Ac	+	-	NT	-	+		-		-		-	
	PCr+Ac	+	-	NT	-	+		NT		NT		-	

NT= Not Tested ; ys= strong yellow product; y= moderate yellow product; and yf= faint yellow product. Qualitative rate of oxygen utilization is given as - <w <+w < + < ++ <+++. Yellow product is indicative of meta-ring-fission.

Recent papers by Keat and Hopper (13, 14, 15) describe several new hydroxylases acting on para-cresol; these enzyme sequences produce intermediates of parahydroxybenzylalcohol and parahydroxybenzaldehyde, which may then be further degraded by as yet uncharacterized enzyme systems. Presumably both ortho- and meta-ring-fission options are open to these intermediates. We have not yet tested our organisms for the presence of these enzymatic actions on para-cresol. But it is possible that the alternate para-cresol pathway in JPT3-4, and the apparent dominant pathway in JPT8, may involve something other than the classic ortho-fissioning of catechol intermediates.

Studies on JPT8 using whole cell suspensions (Table 2) show an apparent lack of a functional meta-cleavage pathway. A number of co-induction tests remain before an absolute lack of induced meta-cleavage enzymes can be proposed. The curious definite but weak oxidation of meta-cresol, coupled with no detectable 3-methyl-catechol (3MCAT) oxidation, seen in para-cresol-induced cells, has several possible explanations which are presented in the Enzyme Studies section. Sonicated cell extracts also show no 3MCAT activity, hence lack of the meta-cleavage pathway continues to be the likely interpretation. Meta-cresol hydroxylase may in fact be induced by para-cresol--they may or may not be the same enzyme -- but the hinderance posed

by the meta-CH_3 group to intra-diol fission might prevent any 3MCAT so made from proceeding via the 4MCAT-active pathway. These and other aspects are peripheral topics of study for continuing investigations.

One reason for high interest in this kind of information has to do with the positive mcre$^+$ Neo^r phenotypes recovered in JPT3-4 x JPT8N mates (Table 3). If JPT8N does possess some or all meta-cleavage enzymes genes, but no competent induction controls involving early intermediates, the induced enzyme picture we presently possess would be misleading. The information crossover completing at T+105 minutes does make JPT8N competent for growth on meta-cresol; but this might be due to crossover of the required structural genes coding for induction functions, rather than due to implantation of functional, enzyme-coding genes. Another possibility is that JPT8 is missing only certain parts of a meta-cleavage pathway, such as the 2,3-oxygenase. In any case, since the crossover is clearly time-dependent, this would argue for a chromosomal loci for at least some functions critical to successful meta-ring-fission of the catechols tested.

Other recipient strains we may use in further interrupted mating and transduction studies have not yet had their induction responses characterized to the extent required for appropriate use. These procedures are also currently underway.

Mating Experiments.

Table 3 presents data obtained from numerous interrupted matings between primary donor JPT3-4 and several recipient strains. None of these have proven ideal, although a pattern does begin to emerge. A major block to progress on this front has been our inability to isolate completely incompetent cresol metabolizers out of many Pseudomonas aeruginosa strains examined. Abilities vary, but apparently enough nonspecific oxygenases are normally present in these organisms to successfully fission the cresol ring, even when a fully-integrated, efficiently controlled enzyme pathway is lacking. This situation, we theorize, is a major factor behind the concentration survival effect: At 100 ppm, many strains may survive without growth, or with only weak growth, on cresols as the sole carbon source; but at successively higher ppm, those with less efficient enzymes systems die out. A second factor here is the probable existence of certain membrane changes that apparently do not directly contribute to substrate metabolism, but rather impact on organism resistance to toxic cresol concentrations. Protective mechanisms could include permeability controls, or membrane protein alterations that inhibit denaturation processes normally caused by high cresol concentrations.

In addition to functional loci coding for the pathway enzymes themselves, there are also structural control sites;

particular control mechanism constellations can make the pathway more efficient in organisms having them than in others which do not. These control mechanisms are also transmissible, and are difficult to detect, although their presence or absence will lead to variable survival capability on the toxic substrate. It is difficult to differentiate between the situation where a functional pathway enzyme gene is communicated into an organism lacking an efficient enzyme, versus the case where a functional gene already exists in the recipient (but with inadequate or inappropriate controls) and an efficient structural gene is transferred in. In the first case, one is introducing the required pathway enzyme itself; in the second, it is the efficient control that is introduced. Both produce the same growth effects. Enzyme induction studies can resolve these cases under certain conditions, but not always.

An example of the complexity of the 'cresol competency' problem is the mate between PAC5 and JPT8. JPT8 grows poorly on meta-cresol BMS agar; PAC5 will also form only tiny colonies on meta-cresol agar when supplemented with histidine. Yet the mate turned out highly competent meta-cresol recombinants. It was not a clear donor-recipient mate (both strains are female) thus the direction of information transfer is unknown. JPT8 can survive high cresol concentrations--though it will not grow in meta isomer broth; PAC5, on the other hand, forms only tiny colonies on 100 ppm

meta-cresol agar, will not grow in meta-cresol broth, and cannot survive 900 ppm concentrations.

Plasmid DNA electrophoresis of JPT3-4 x JPT8N recombinants showing the PMO^+ phenotype yielded the patterns exhibited in Figure 2. Note that although some recombinants now possess the JPT3-4 90 Md plasmid (further described in DNA Studies, below) there are some which do not. Additional plasmid bands are in evidence which were not visible in either parent, notably some very low molecular weight bands near the bottom of the gels. Since there is no consistency in the selection for these plasmids under the experimental conditions, one can assume that they do not code for information critical to JPT8N's survival on 100 ppm meta-cresol/neomycin agar, the selecting substrate. This does not rule out the possibility that other structural or functional loci involved with survival on and utilization of the cresols may reside on one or more of these extrachromosomal entities.

TABLE 3.

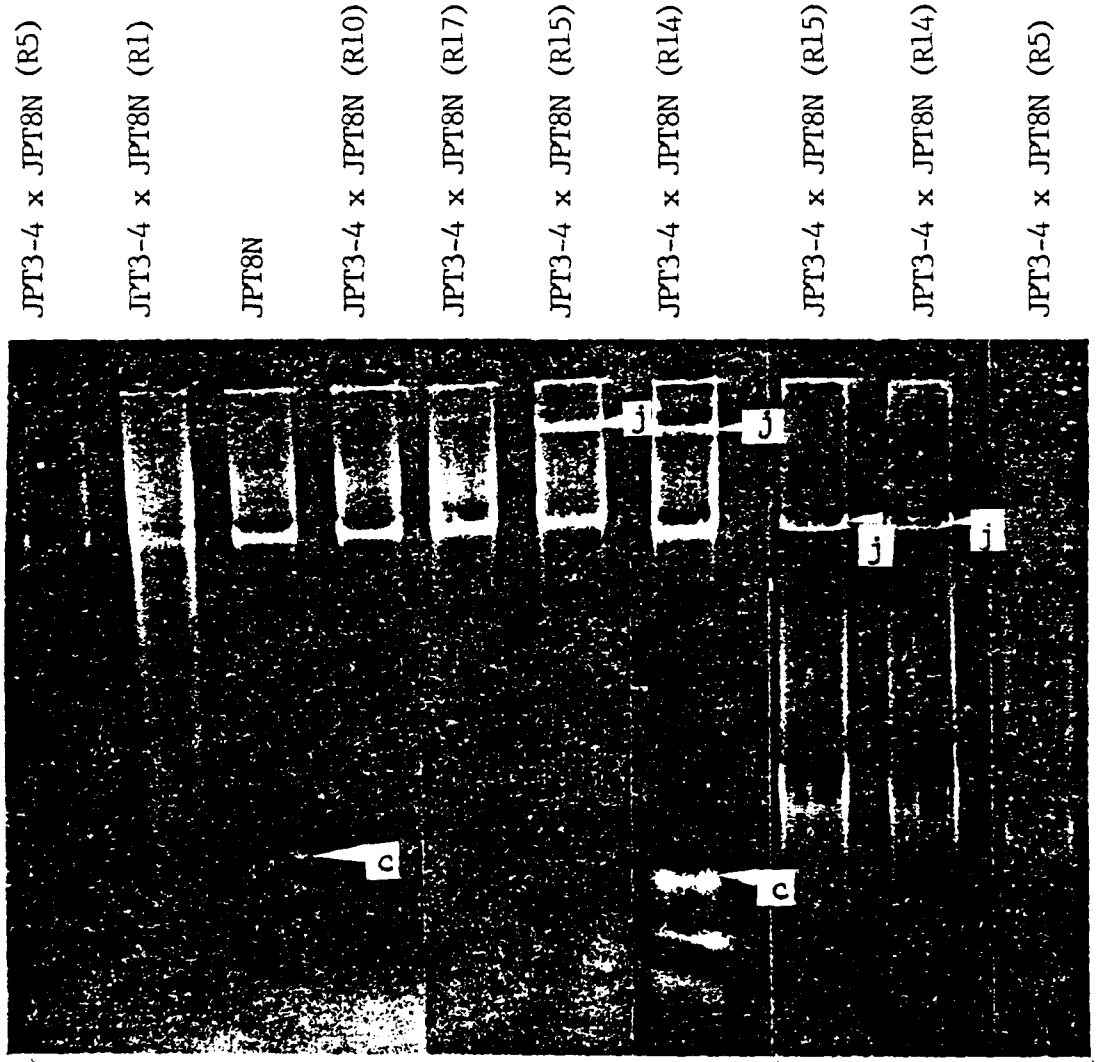
INTERRUPTED MATING DATA**

		Interrupt Time (minutes)									
Donor X	Recipient	5	15	30	60	75	90	105	120	180	240
JPT3-4	PAC5SR	0	0	0	$1.6X10^{-8}$	NT	NT	NT	$2.6X10^{-3}$	NT	NT
JPT3-4	PAO222	0	0	0	0	0	0	NT	NT	NT	0
JPT3-4	JPT8N	0	0	0	0	$4X10^{-8}$	$4X10^{-8}$	$3X10^{-3}$	$0.5X10^{-3}$	NT	NT
JPT8N*	PAC5*	NT	NT	NT	NT	NT	NT	NT	$0.3X10^{-4}$	NT	NT

* Information transfer direction unknown.
** Selection made on M100 Neo agar.

FIGURE 2

DNA Studies of Mating Recombinants



3mm 0.7% agarose gels in E-buffer

[j] = JPT3-4 plasmid band

[c] = cryptic plasmids

DNA Studies.

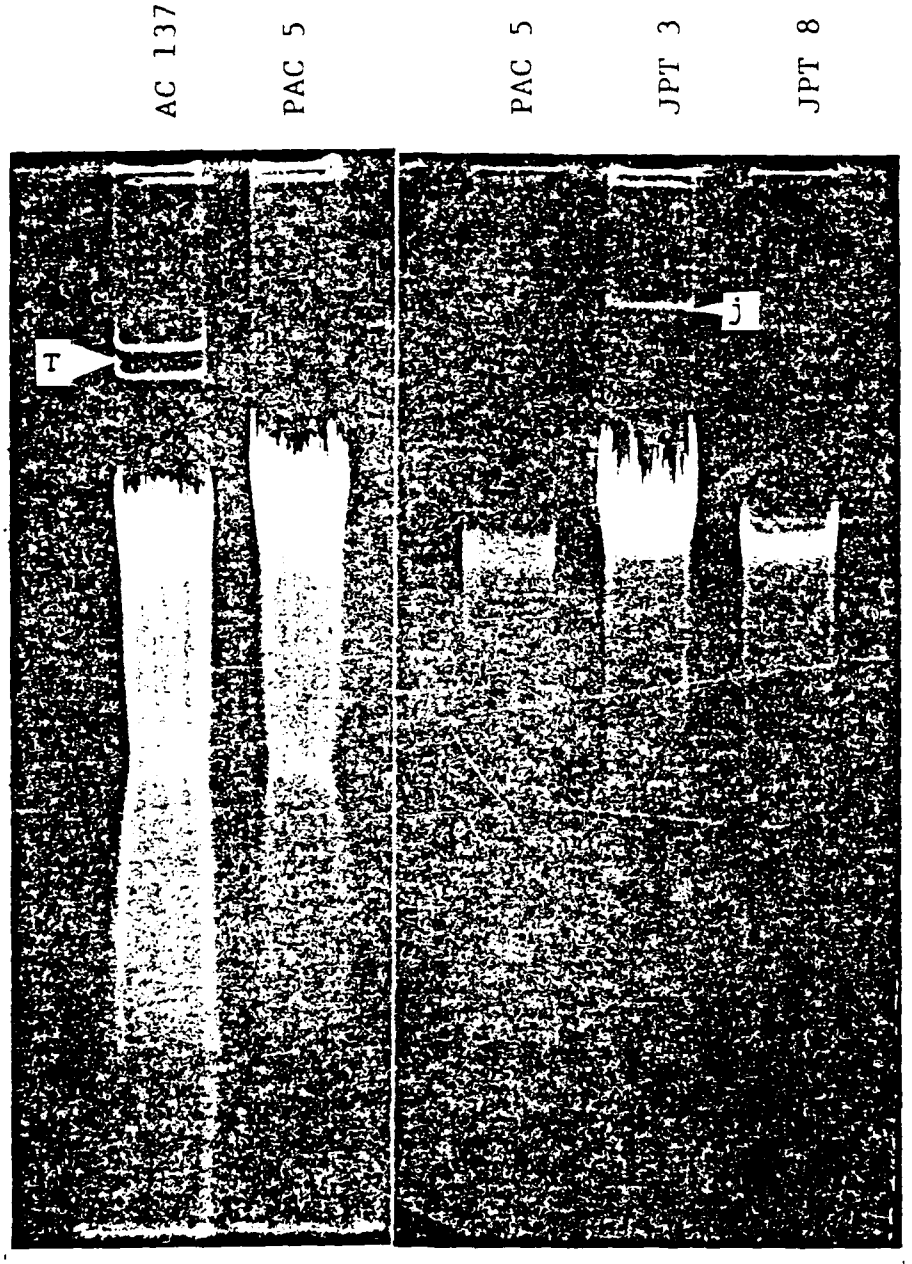
A variety of DNA-extraction techniques have been applied to the strains listed in Table 1 , primarily designed to extract cytoplasmic (plasmid) DNA. The methods of Guerry et al. (16), Humphreys et al. (17), and Hansen and Olsen (18) have been evaluated. Although no significant differences in plasmid population resolution have been detected between the three methods, Hansen's and Olsen's alkaline denaturation PEG-precipitation method has yielded by far the cleanest preps with all Pseudomonas and Corynebacterium species tested. Using Meyers' electrophoresis technique (19), plasmid DNA bands have been visualized from both parent and recombinant species. The TES-DNA suspension extraction product has been used in transformation studies.

In addition to several low copy number plasmids which remain cryptic at this writing, a band associated with JPT3 and its Str^S (streptomycin sensitive) variant, JPT3-4, and their $PMO\emptyset^+$ offspring, has been visualized (Figure 3). This band is absent in the JPT8 $P^+MO\emptyset^-$ strain. Compared to TOL of AC137, JPT3-4's plasmid is heavier at approximately 90 Md.

Transformations using crude plasmid DNA extract from JPT3-4 were attempted using the method of Cohen et al. (20) on Chakrabarty's P. putida AC137, which harbors a TOL plasmid coding for meta-ring-fission enzymes for

FIGURE 3

JPT3 and JPT8 Plasmid Populations



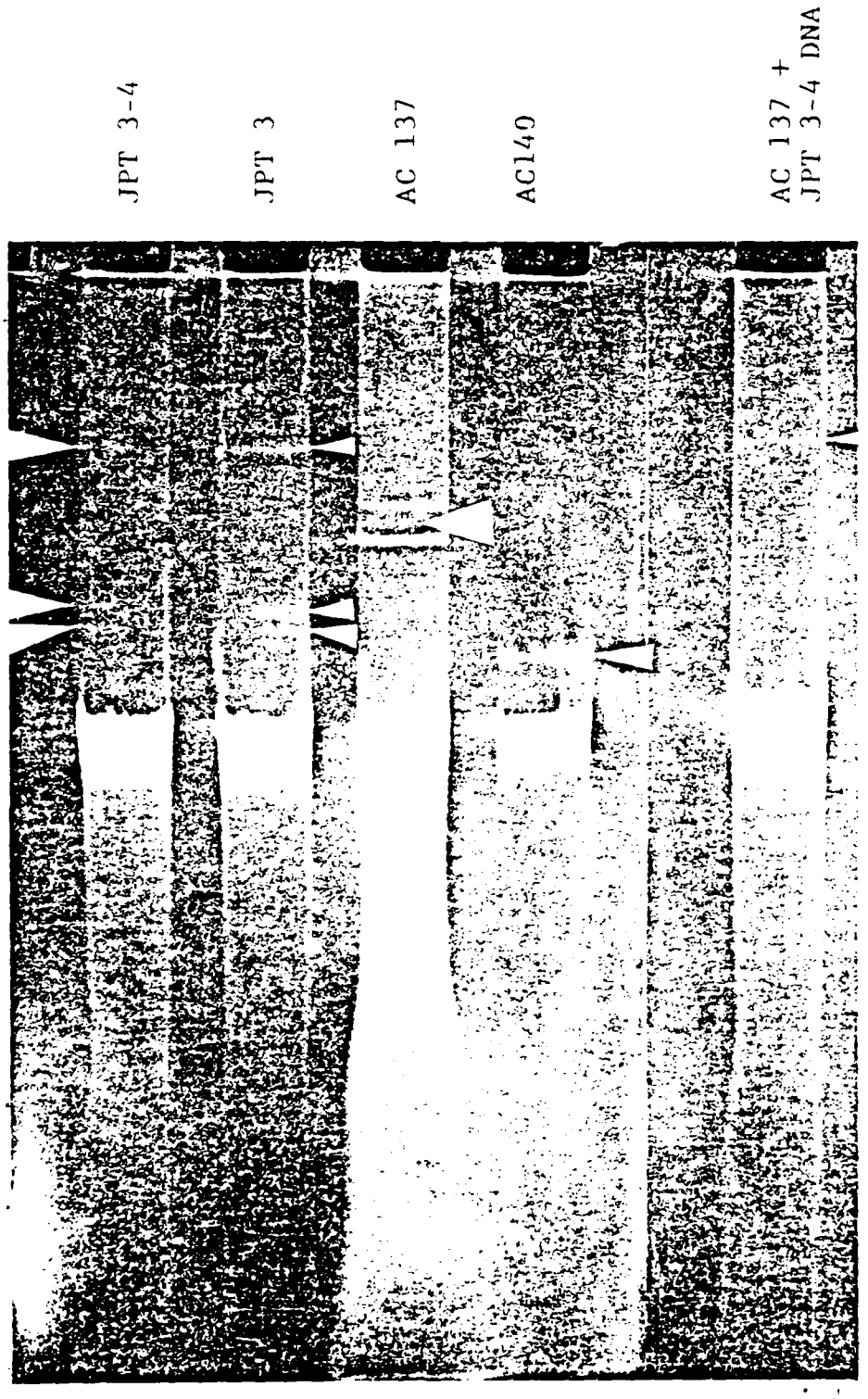
0.7% Agarose 120V 60ma, double 3mm gels 2 hours

[j] = JPT3 (and JPT3-4) plasmid

[T] = TOL

toluene and xylene dissimilation (A.M. Chakrabarty, personal communication). AC137 is a *met*$^-$ auxotroph, and was determined to be $\emptyset^+$ and PMO$^-$ at 900 ppm. However, it did show weak competency on para-cresol BMS agar at 100 ppm, supplemented with methionine. Transformants were obtained which demonstrated no meta-cresol capability, but which were better capable of growth on para-cresol/ methionine minimal agar. In these transformants, the TOL plasmid was missing, apparently supplanted by the plasmid from JPT3-4 DNA (Figure 4). This incompatibility with TOL places it in the P-9 incompatibility group (10).

Exactly what functions were selected in this transformation is unclear, since the *mcre*$^+$ phenotype did not appear. TOL$^-$AC137 transformants still showed $\emptyset^+$ capability, but without enzyme information one cannot draw conclusions about any changes in pathways that may or may not have occurred as a result of the TOL plasmid's supplantation. If TOL is the only set of genes conferring meta-ring-fission capability on AC137, such pathway changes might be expected, unless the supplanting plasmid also codes a suitable set of enzymes. Still another possibility is that the supplanting plasmid in fact carries no cresol degradation-involved genes at all, but is incompatible with TOL, ejecting TOL and thereby allowing a chromosomally-encrypted, TOL-repressed pathway to operate. Such a situation has actually been observed in the case of benzoate



0.7% Agarose 120V 73ma double 3mm gels 2h 30m

FIGURE 4

TOL Plasmid and JPT3-4 Plasmid
Interaction

oxidation, wherein it was found that the chromosomal ortho-fission enzymes acted more efficiently than the TOL-coded benzoate oxidase activity, but was repressed in the presence of TOL (6).

The thrust of current enzyme studies is to combine enzyme and oxygen probe induction data in tracking the effects of recombination and transformation on cresol-isomer metabolism. Baseline data for donor and recipient strains may allow a clearer view of what is being transferred in successful recombination and transformation events.

Enzyme Studies.

Initial investigation of the J1 meta-ring-fission was accomplished in 1977-78 (12). Coupled with O_2-electrode studies on whole cells, a complex control over cell permeability to meta- and ortho-cresol was implied. This year, difficulties were encountered in obtaining active preps when the high pressure hydraulic press at a distant teaching hospital was used for mass cell lysis. After a Lab-Line/Wave Energy Systems Model MF1 Sonicator was obtained, renewed efforts were begun to examine enzyme levels in key organisms. It was observed that the phosphate buffer conventionally used in our preps seemed to yield rather low levels of activity. Similar observations recorded by Ornston and Stanier (21) prompted trials with a new Tris-HCl/Mg-EDTA buffer using a procedure described by Chakrabarty (8). These trials have not yet been completed.

Premilinary results from preps of JPT3-4 and J20 support the existence of at least two pathways for Pseudomonas JPT3-4 (ortho- and meta-ring-fission), and appear to rule out any coinduced meta-cleavage pathway in Corynebacterium J20 when the organism is grown on phenol. Oxygen electrode studies on enzyme preps have been initiated for JPT3-4 and JPT8, giving results listed in Table 4. It is noteworthy that the pathway induced by growth on para-cresol apparently contains

enzymes acting almost as well on protocatechuic acid (PROCAT) as on 4-methyl-catechol (4MCAT), but showing essentially no activity on either catechol (CAT) or 3-methyl-catechol (3MCAT). Whole cell data, as mentioned in the Oxygen Electrode Studies, did show positive activity on meta-cresol, which is not a growth substrate for JPT8; but since the organism has no detectable induced 2,3-oxygenase, so far as we have determined, the 3MCAT created by the hydroxylase step is very likely excreted. (Actually, marginally surviving colonies are formed by JPT8 on meta-cresol agar, indicating either a very low level of 2,3-oxygenase activity, or--far more likely--the extremely inefficient ortho-fissioning of the sterically-hindered intra-diol ring bonds by the same oxygenase(s) which act so efficiently on 4MCAT and PROCAT.

The Corynebacterium J20, which we had studied along with the Pseudomonads in earlier studies, expresses an extremely efficient pathway similar to that found in JPT3-4, but with different induction controls. Phenol-induced J20 yields enzyme activity on the catechols with peaks at 257-260 nm (Burkholz, unpublished data), indicating ortho-fission of the catechol nucleus; JPT3-4 on phenol shows signs of having both meta- and ortho-fission paths. An interesting aspect of C. J20's metabolism is its ability to use para-, meta-, ortho-cresol, phenol, toluene and toluate, and benzoate as sole carbon sources, at extreme

concentrations--to 1300 ppm without inhibition--and its extraordinary resistance to high hexavalent chromium levels--up to at least 59 ppm (3).

Further baseline studies of JPT3-4, JPT8, J20, and selected recipient and recombinant strains' enzyme activities and induction specificities are currently in progress.

TABLE 4

O_2-PROBE ENZYME RESULTS, PRELIMINARY

STRAIN	INDUCING SUBSTRATE	TEST SUBSTRATE PCr	MCr	Ø	Bz	4-M-Cat	3-M-Cat	Cat	Pro-Cat
JPT3-4	PCr	NT	NT	-	-	+	w	-	+++
JPT8N	PCr	-	NT	NT	NT	w	-	+	+++

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