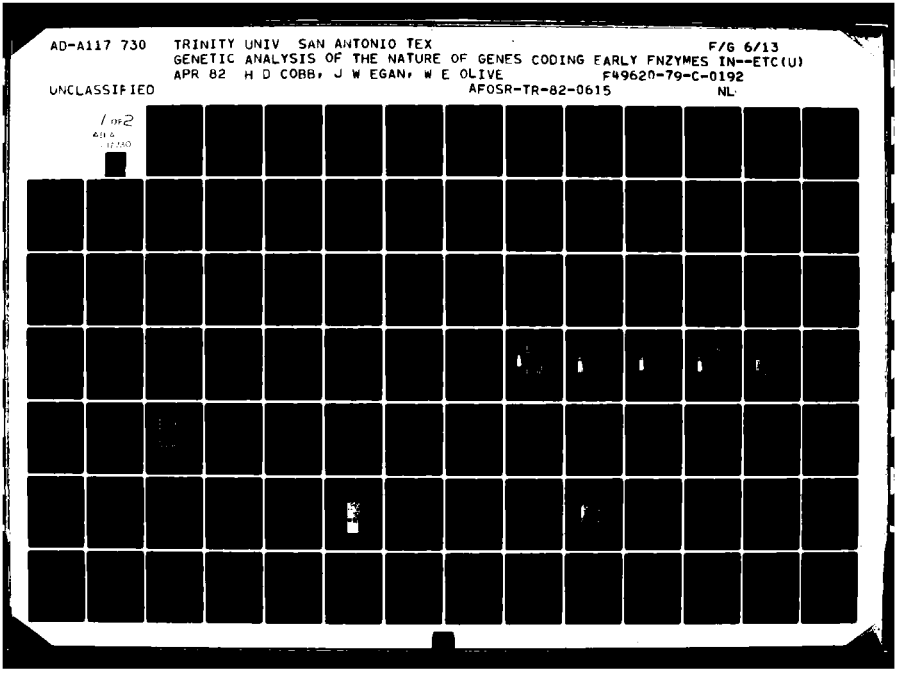
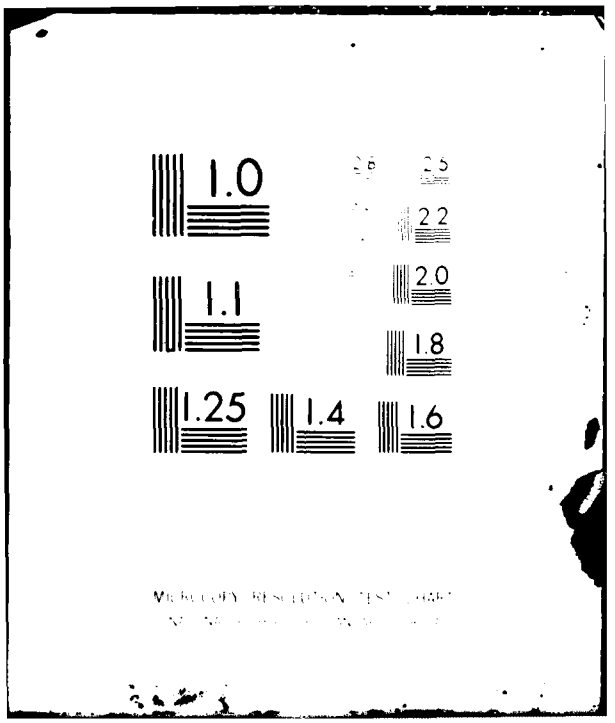
AD-A117 730 TRINITY UNIV SAN ANTONIO TEX F/G 6/13
GENETIC ANALYSIS OF THE NATURE OF GENES CODING EARLY FNZYMES IN--ETC(U)
APR 82 H D COBB, J W EGAN, W E OLIVE F49620-79-C-0192
UNCLASSIFIED AFOSR-TR-82-0615 NL




1.0
1.1
1.25
1.4
1.6
2.2
2.0
1.8

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Ente

(12)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER **AFOSR-TR- 82-0615**	2. GOVT ACCESSION NO. AD-A117730
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle) Genetic analysis of the nature of Genes coding early enzymes in the metabolism of Cresol in JPT3-4, a derivative strain of Pseudomonas aeroginosis J1	5. TYPE OF REPORT & PERIOD COVERED Final 79-81
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Howell D. Cobb, Jr., Ph.D. John W. Egan, Jr. William E. Olive	8. CONTRACT OR GRANT NUMBER(s) F49620-79-C-0192
9. PERFORMING ORGANIZATION NAME AND ADDRESS Trinity University 715 Stadium Dr. San Antonio, Texas 78216	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102F 2312/A5
11. CONTROLLING OFFICE NAME AND ADDRESS Air Force Office of Scientific Research Bolling AFB, DC 20332	12. REPORT DATE 4-29-82
	13. NUMBER OF PAGES 107
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report) UNCLASSIFIED
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release;
distribution unlimited.

DTIC ELECTE AUG 0 3 1982 S E

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

Presented at the 1982 meeting of the American Society for Microbiologists

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Genetic Analysis of Genes involved in Metabolism of Cresol

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A pseudomonas strain, JPT3-4, was developed which was competent on all three cresol isomers (para, ortho, and meta). This organism maintained a transmissable FP type plasmid which could transfer the genes required to metabolize cresol to cresol incompetent strains of related species. The transfer of these plasmids was visualized via gel electrophoresis and the metabolic activity of the recipient strains was followed through the use of an oxygen electrode and spectrophotometric analysis of their metabolites.

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

AD A117730

DTIC FILE COPY

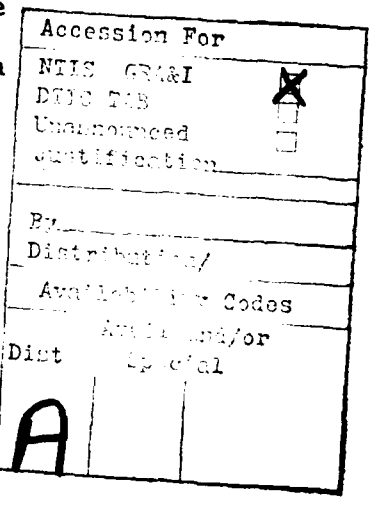
AFOSR-TR- 82-0615

FINAL REPORT

ON

AFOSR CONTRACT F49620-79-C-0192

Genetic Analysis of the Nature of Genes Coding Early Enzymes in the Metabolism of Cresol in JPT3-4, a Derivative Strain of Pseudomonas aeroginosa J1.



Approved for public release; distribution unlimited.

82 08 03 119

INTRODUCTION

The past two decades have seen increased application of microbial metabolism to waste management problems (Metcalf and Eddy, Inc., 1972; Lanouette, 1977), an aspect of technological innovation with roots in the prehistoric development of microbially-processed foodstuffs. Initially it was the low level of engineering technology involved, coupled with economical operating costs, that made biological degradation of industrial wastewaters an attractive treatment alternative (Hawkes, 1963; Behn *et al.*, 1968). Inasmuch as phenolic wastes had by 1960 become a major component of large-scale industrial treatment facility influents, many studies centered on the kinetics of phenol biodegradation in various reactor schemes (Helmers *et al.*, 1952; Huber, 1967; Wurm, 1968; Kostenbader and Flecksteiner, 1969; Nakashio, 1969; Rao, 1969; Radhakrishnana and Ray, 1971). Then public awareness of the mushrooming industrial pollution problem mobilized local and federal governments to increase enactment of pollution-control legislation and brought about an unprecedented degree of self-examination among the pollution producers in both the private and public sector (U.S.E., , 1977). The U.S. Air Force was one of the first federal agencies to fund a multi-faceted investigation into the extent of their production of industrial toxic wastes (Brewer *et al.*, 1968; Fishburn *et al.*, 1970; Kroop, 1973; Cobb *et*

al., 1974; Perrotti, 1975). The Air Force's ultimate objective was a cost-effective treatment method for handling the wide range of industrial wastes produced at varying scales at the main Air Logistics Command Centers (ALC's).

It was our contention that an adapted ecosystem of organisms with a complementary metabolic network centered on particular toxic compounds might improve on the current efficiencies of industrial waste biological reactors (see Ingold, 1940; Bushnell and Hass, 1941; Barker, 1949; Helmers et al., 1952; Hawkes, 1961; Brebion et al., 1963). The three-year study began in 1972 with the enrichment isolation of numerous strains of bacteria from the paint-stripping room in Bldg. 375 at Kelly Air Force Base (KAFB), the San Antonio, Texas Air Logistics Command Center (ALC); and concluded with studies of the efficiencies of cresylic acid degradation by strains selected from those isolates in a laboratory-scale pilot plant at Trinity (Cobb et al., 1974; Mitchell, 1974; Olive, 1975; Olive et al., 1976).

Cresylic acid--a 1:1:1 mixture of the three isomers of cresol--was a major component of the paint-stripping compound.

Hatcher's observations indicated that the ability to degrade the meta- and ortho-cresol isomers could apparently be lost from a population of cresol-growers raised on rich carbon sources and stores in the cold (Cobb et al., 1977). At the time, another proposal was in the works, one which was to push forward with the application of the J-series isolates as primary phenolics

degraders in an ecosystem "genetically tailored" to the KAFB-ALC waste stream. We became concerned that an instability in the J-series' metabolic traits might at some point pose a problem in the operation of a biodegradation reactor.

Hatcher's metabolic studies of Pseudomonas aeruginosa J1 yielded some curious results which later proved significant in unraveling the nature of J1's metabolic instability (see DISCUSSION). In addition, a 1975 set of preliminary experiments by Olive (personal communication), wherein he had treated J-series isolate, J4, with mitomycin-C, an agent previously used by Chakrabarty's group for "curing" a Pseudomonas putida of the CAM plasmic (Rheinwald et al., 1973), had yielded numerous "cured" strains. One of these, strain J4-8, was capable of metabolizing the para isomer of cresol, but neither the meta nor ortho isomers; whereas parental strain J4 had efficiently metabolized all three cresol isomers. Hatcher had shown that meta- and ortho-cresol were degraded in the J-series pseudomonads principally by the so-called "meta-fission" or 2,3-oxygenase (extra-diol fission) pathway. From these findings, it was hypothesized that in the J-series pseudomonads, at lease some of the genes critical to the "meta-fission" pathway might be plasmid-borne. Our task became one of examining the tenability of that hypothesis, within the context of the much more pragmatic Air Force objective of assuring the stability of cresol metabolism in a "tailored" microbial ecosystem. In practical terms, our objective was to determine the loci of genes reponsible for

critical aspects of cresol degradation in a J-series pseudomonad: i.e., were plasmic or chromosomal genes involved? And second, what might be a practical means of stabilizing those apparently unstable genes affecting cresol metabolism in J1? The results of this effort are presented in this report.

Specific Aims

The specific aims of this work are to:

(1) establish the primary enzyme pathways induced in two bacterial isolates, *Pseudomonas aeruginosa* JPT3-4 and JPT8N, during their growth on phenol, the three cresol isomers (para-, meta-, and ortho-cresol), and benzoate;

(2) establish baseline induction data for the hydroxylase and oxygenase systems in the main phenol and cresol catabolic pathways in the two test organisms, and compare these results to those obtained for their recombinants;

(3) visualize the plasmid populations of both test organisms and their recombinants, and compare to other organisms, both those lacking and those demonstrating similar catabolic pathways, using a variety of DNA extraction techniques;

(4) suggest the probable nature of critical factors responsible for the ability of these organisms to exist on high concentrations of phenol and cresols as sole carbon sources; and

(5) suggest the genetic location of information coding for the hydroxylase and oxygenase steps in aromatic ring-fission of phenol and cresol isomers in these organisms.

METHODS AND MATERIALS

Experimental Design

In order to arrive at a better understanding of cresol metabolism in the J-series pseudomonads, this study was designed to investigate cresol metabolism on a relatively wide front compared to the earlier work by Hatcher in this laboratory (Hatcher, 1977), with special emphasis given to the genetic aspects. Three principal topics for experiment were outlined:

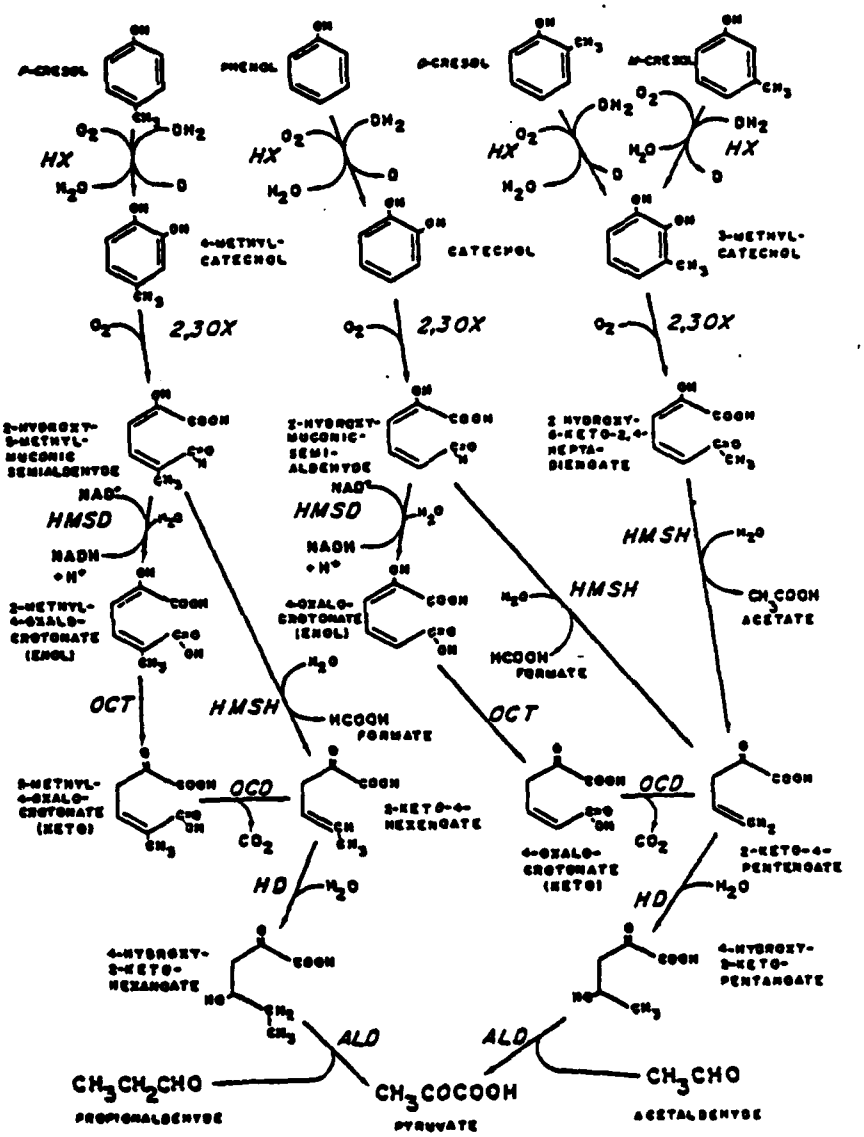
(1) *Gene expression in mate exconjugants.* The ability of a cresol-competent donor strain to transfer its *cre*$^+$ phenotype to incompetent or partially competent recipients was examined. In long-term mates (24 hours) of recipient and donor, qualitative indications of recombination could be observed. Using the interrupted mating technique of Jacob *et al.* (1960) and Jacob (1966), indications of linkage of the *cre*$^+$ phenotype to transfer of donor chromosome markers was detected; yet in matings with certain other recipients, there was evidence that linkage was only partial. This ambiguity indicated that other more subtle means were required for a more complete interpretation of the conjugation studies. The screening of mate "exconjugants" through replicaplatings onto selective media would serve as the initial step in the observation of *cre* gene expression.

(2) *Enzyme level changes.* Baseline studies were done to obtain induction control fingerprints for the donor and recipient organisms involved in matings. These baseline studies involved both cell extracts as well as whole-cell preparations. Cell extracts were examined primarily in the spectrophotometer to qualitatively characterize their 1,2- and 2,3-oxygenase activities on the catechols derived from the cresols through hydroxylation (see Figures 1 & 2). Whole-cell preparations were used in the oxygen electrode to record both the combined activities of the cresol hydroxylase(s) and oxygenase(s); and to examine the activities of the oxygenase(s) alone on the catechol substrates. Comparison of activity levels in an organism raised on different combinations of cresols, phenol, benzoate, and a non-phenolic carbon source (acetate or succinate), gave insight into the induction control mechanisms present. Comparison of baseline data with similar tests on selected exconjugants--the putative recombinants--gave some idea of the distinctness of gene loci responsible for those controls.

(3) *Cell plasmid populations.* Inasmuch as the initial aim of the study was to determine if the plasmid nature of the meta- and ortho-cresol degradation genes suggested in the curing experiments could be supported, the plasmid populations of cresol-competent cells were isolated and mate exconjugants were also thoroughly studied to check for linkage between the

FIGURE 1

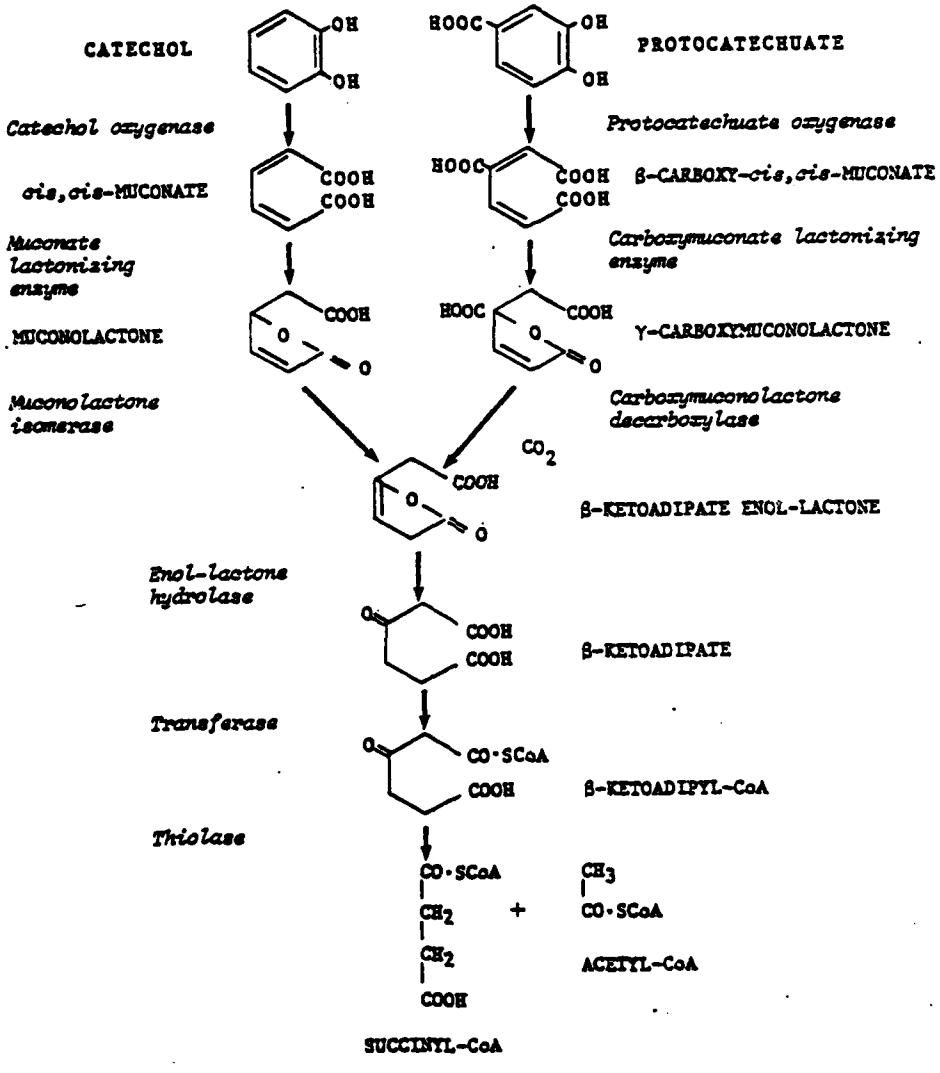
"META" CLEAVAGE OF CRESOLS AND PHENOL



These pathways were shown to operate in *Pseudomonas aeruginosa* J1 by Hatcher (1977). Only the hydroxylating and oxygenase ring-fissioning activities are examined in this report. (After Hatcher, 1977.)

FIGURE 2

"ORTHO" FISSION PATHWAYS FOR PHENOLS AND PROTOCATECHUATE



(After Clarke and Richmond, 1975.)

presence of a plasmid and cresol competency. As later experiments showed, it was also necessary to check for competency on the catechols alone. Several techniques of plasmid DNA isolation were investigated. Transformation of cresol-incompetent organisms by plasmid DNA from a cresol-competent strain was also attempted, and transformants examined.

Many of the experiments reported, especially in the earlier stages of the study, yielded tentative or negative results. Isolation of both competent donors and recipients proved to be a difficult process, with unequivocal success coming only in the last nine months of this study. However, the experimental design as outlined has resulted in the establishment of a key data base for the donor and recipient organisms; the identification of a previously unreported membrane-associated 2,3-oxygenase system of extreme efficiency and good durability together with the development of a suitable extraction technique; and the localization of some of the critical genes involved in cresol metabolism. Computer programs for organizing and reducing data from spectrophotometric and oxygen polarographic studies have also been developed and are available for further application.

Organisms and Methods of Cultivation

Stock cultures of *Pseudomonas aeruginosa* J1, J2, J3, J4, J4-8, PAO222, PT013, and PAO25 were revived from long-term storage on tryptic soy agar slants at -40°C. The J-series organisms had been isolated by Olive (1976) from the paint-stripping facility at Bldg. 375, Kelly AFB, as part of a program to develop a microbial ecosystem able to degrade Kelly paint-stripping wastes. The last three organisms listed were the kind gift of Dr. Eric Moody of the University of Texas Health Science Center at San Antonio, and were originally wound-isolates first introduced into genetic studies by Holloway (1955 and 1969). All three were amino-acid auxotrophs; PT013 carried the FP2 sex factor plasmid. *Pseudomonas aeruginosa* AC140 (a *met*$^-$ *ami*^{del} strain) and *Pseudomonas putida* AC137 (a *met*$^-$ strain), both carrying the TOL (toluene-degradation via "meta-fission") plasmid of Williams and Murray (1974), were obtained courtesy of A.M. Chakrabarty, then at General Electric's Schenectady, N.Y., research facility. Several other non-cresol-degrading strains of *Ps. aeruginosa* and *Ps. putida*, most carrying chromosomal deletion markers, were obtained from various sources through the kind efforts of Dr. James Walker of the University of Texas at Austin Department of Microbiology. For a complete list of all strains involved in these studies, their growth substrates

of interest, markers, sources, and plasmid populations, see Table 1.

The principal donor strain, JPT3-4, was a derivative initially selected from a long-term (24 hr.) mate in Luria broth (Pemberton and Clark, 1973) between strains J1 and PTO13. Selection was for a *Pcre*$^+$ (para-cresol-degrading) prototroph on 900 ppm para-cresol basal mineral salts (BMS) 2% agar--written as P900--followed by replicaplating onto similar media containing 900 ppm of the meta (M900) or the ortho (O900) isomer. Examination of *PMOcre+* colonies so detected for the presence of an FP2 plasmid band from PTO13 (see *Plasmid DNA Extraction Techniques*, and also *Mating Procedures*, below) yielded the male *PMOcre*$^+$ FP2$^+$ JPT3. Final isolation by replicaplating onto tryptic soy-Streptomycin media (TSS, consisting of standard tryptic soy agar supplemented with 1000 ppm Streptomycin hydrochloride), and onto tryptic soy-Neomycin media (tryptic soy agar with 400 ppm Neomycin sulphate), allowed the selection of a *PMOcre*$^+$ FP2$^+$ *Str*^{s} *Nm*^{s} (antibiotic sensitive) donor strain, JPT3-4. From the same J1 x PTO13 mate, a *Pcre*$^+$ *MOcre*$^-$ FP2$^+$ *Str*^{r} *Nm*^{r} (antibiotic resistant) recipient strain, JPT8SRN, was isolated. These two organisms became the principal objects of study for subsequent genetic experiments.

TABLE 1

BACTERIAL STRAINS

STRAIN	GENUS	SPECIES	SOURCE	PHENOLIC GROWTH SUBSTRATES	MARKERS	PLASMIDS
J1	*Pseudomonas*	*aeruginosa*	KAFB-ALC, Bldg 375	*PMOcreØBzTol*$^+$	None	FR*
J4-8	"	"	J1/Mitomycin-C cure	*PcreBz*$^+$	None	FR*
PTO13	"	"	Moody, UTxHSC	None	*trp6*	FP2
JPT3	"	"	J1 x PTO13	*PMOcreØBzTol*$^+$	None	FP2
JPT3-4	"	"	Selected JPT3	*PMOcreØBzProcatTol*$^+$	*Nm*^{S}	FP2
JPT8	"	"	J1 x PTO13	*PcreBz*$^+$	None	FR*
JPT8N-80	"	"	Selected JPT8	*PcreBzProcat*$^+$	*Nm*^{r}	FR*
PAS102	"	"	Meyers & Walker	None	*thr102*	None
PAS102N	"	"	Selected PAS102	None	*thr102 Nm*^{r}	None
PAO222	"	"	Moody, UTxHSC	None	*his*$^-$*trp6 met28 pro82 lys12 ilv 226 StrA*	None
AC137	*Pseudomonas*	*putida*	Chakrabarty	*ØTol*$^+$	*met*$^-$	TOL-type
J20	*Corynebacterium*	*spp.*	KAFB-ALC, Bldg 375	*PMOcreØBzTol*$^+$	None	None

*FR = "front runner" band, labelled by other investigators as "linear DNA fragment," and "chromosomal DNA fragments with an upper size limit." (See text sections on plasmid DNA preparations.)

PMOcre = para-, meta-, & ortho-cresol; *Ø* = phenol; *Bz* = benzoate; *Procat* = protocatechuate; *Tol* = toluate. *Nm*^{S} = Neomycin-sensitive; *Nm*^{r} = Neomycin-resistant; *trp* = tryptamine; *thr* = threonine; *his* = histidine; *met* = methionine; *pro* = prolamine; *lys* = lysine; *ilv* = isoleucine-valine; *StrA* = Streptomycin resistance.

Maintenance of stock cultures. "Working stock" cultures were maintained at room temperature (about 26°C.) on 2% BMS agar with appropriate carbon sources. Cresol degraders of the J-series were kept on media containing either 900 ppm or 100 ppm of a cresol isomer, supplemented with 400 ppm Neomycin sulphate or 1000 ppm Streptomycin hydrochloride where appropriate. Auxotrophic strains were maintained on Luria agar, with antibiotics added where appropriate; these cultures were checked by replicaplating isolated colonies onto succinate or acetate BMS agar and onto succinate or acetate supplemented with the required amino acid(s) before their use in mating procedures, to establish reliability of the chromosomal deletion markers. Room temperature stocks were transferred to fresh slants of the same type at three week intervals, or at the time the particular culture tube was used as an inoculum source.

"Long term stock" cultures were banked by growing for several days at room temperature on Luria agar slants, then storing them under sterile mineral oil at -40°C. in sealed test tubes. (Mineral oil may be sterilized by autoclaving, followed by air cooling in a container with a loosened top to prevent formation of a vacuum and to allow suspended moisture to separate.) Freeze-banked stocks were revived for use by streaking onto Luria agar plates and incubating for 3 to 4 days before transferring colonies to a Luria grid plate for replicaplate testing for growth on specialized media. Particularly for high

concentration cresol growers, this recovery period was a necessity (see DISCUSSION). Revived colonies meeting identification criteria were then entered into the working stock for experimental use. Normally, freeze-banked cultures were used as sources only when working stocks became contaminated or were otherwise lost. (A troublesome infestation of phenol-resistant mites from a neighboring experimental culture stock proved the value of the freeze bank on numerous occasions.) Long term stock tubes used for reestablishing working stock were refrozen, along with a freeze bank tube of the newly-reestablished culture. Stocks maintained in this fashion have proven viable for over three years.

Culturing for mating experiments. Donor organisms were taken from working stock slants, normally M900 or 0900 media, inoculated into 50 ml of 2-day-old Luria broth (free of contaminants), and incubated as a stationary culture at 28°C. for 15 to 18 hours. The recipient strain, taken from its working stock slant--which normally contained the appropriate antibiotic selection "handle"--was also inoculated into 50 ml of 2-day-Luria broth, then incubated on a New Brunswick rotary shaker at 150 rpm at 28°C. for 15 to 18 hours. One hour prior to mating, 25 ml of each culture was pipetted into nephelometer flasks containing 25 ml of fresh Luria. Immediately prior to mating the optical density (OD) at 600 nm of each

culture was determined with a Coleman Jr. spectrophotometer, and used to estimate cell density by consulting a previously calibrated turbidimetric chart. Then optical density was adjusted by adding sterile Luria broth until donor cultures contained approximately 10^8 cells/ml and recipient cultures 5 x 10^8 cells/ml. Five ml of each culture were then dispensed into a set of 25 ml Erlenmeyer flasks: one control flask per parent, each containing 5 ml of sterile Luria to yield a dilution similar to that obtained in the mating vessels; and as many empty 25 ml Erlenmeyer flasks as interrupt times scheduled for the mate. The mating procedure was then promptly begun.

Culturing for whole cell oxygen consumption experiments. Since strain JPT3-4 could utilize all three isomers of cresol, phenol, and benzoate as sole carbon sources with very high growth rates, the growth substrates and inducing substrates for this organism were one and the same. However, JPT8SRN was unable to grow on meta-cresol, ortho-cresol, or phenol alone; combinations of these non-growth compounds were used with either para-cresol, benzoate or acetate as the carbon source. Similar combination inducer/carbon source trials were run on JPT3-4 to compare results, yielding some interesting insights into induction controls. For cresol-incompetent strains, such as PAS102N, succinate plus required amino-acid (1000 ppm and 40 ppm, respectively) served as growth substrate, with inductive concen-

trations of the phenolic compounds (250 ppm) added either at the time of inoculation, or, if the compound proved too toxic, added after growth, some two hours prior to harvest.

Cultures were grown in one liter spinner flasks. Carbon sources were initially present at 500 ppm for the phenolics (total), 1000 ppm for acetate or succinate, and 40 ppm for any required amino-acids, in a BMS buffer base. Growth to late mid-log phase normally required 48 - 72 hours, with carbon source feedings of 250 ppm twice a day. On the day of harvest, cultures were fed carbon source and inducer(s) at 250 ppm two hours prior to harvest. Harvesting was by centrifugation, first in 250 ml polyethylene bottles in the GSA rotor of a Sorvall refrigerated centrifuge at 4000 g at 0°C., followed by two washes using cold BMS buffer with centrifugation in the SS34 rotor at 5000 g. (See DISCUSSION for comments on the use of cold buffer.) The cell pellet was normally split at this point for use in both the oxygen probe experiments and in the enzyme assay procedures.

Culturing for enzyme extraction and activity experiments. Procedures for cell culturing for the enzyme experiments were identical to those for oxygen electrode tests. It was a principal aim of these studies to determine the correlation of the two types of assay techniques.

Other methods of cell preparation for enzyme assay

were also tried. Hatcher (1977), patterning his methods after those of Bayly *et al.* (1966), had utilized a pH 7.5 14.7 mM KH_2PO_4 buffer for washing and storage of J1. His technique, which involved the homogenation of cell pastes in a Gaulin hydraulic homogenizer, required large volumes of cells. The technique eventually developed in the present study was able to obtain high activities from relatively small volumes (2 to 5 grams) of cell paste. Other experiments indicated that the use of cold BMS washes, allowing simultaneous preparation of cells for the oxygen electrode studies, did not affect recovered activity, although a 0.05M KH_2PO_4/Na_2HPO_4 buffer, adjusted to pH 7.5 with NaOH, yielded better results as the suspending agent in the actual sonication and spectrophotometric procedures (*q.v.*).

Culturing for plasmid DNA extraction. Isolated colonies were inoculated from selective agar slants or plates into 150 ml of Luria broth for overnight growth at 30°C. on a New Brunswick rotary shaker at 150 rpm. Short-term culture tubes of selective media were also inoculated, in case the organism should prove worth follow-up study. Cultures were harvested in late mid-log phase in 250 ml polyethylene bottles in the SS34 rotor at 5000 g for 10 minutes at 0°C. After two cold BMS washes the pellets were ready for DNA extraction.

Media Preparation

Buffers. For media purposes, the basal mineral salts (BMS) buffer of Olive (1975) was used. This buffer was also utilized in oxygen electrode experiments with whole cells. In enzyme experiments, both phosphate and TRIS-based systems were tried. Details are contained in the section on enzyme testing procedures, below.

Broth vs. agar media. In all cases, the agar media for a given recipe was identical to the broth recipe plus 20 grams per liter of Difco Bacto-Agar.

Tryptic soy and Luria broths. Tryptic soy was prepared with the Difco dehydrated Bacto-Tryptic Soy Broth with Dextrose formulation, containing 17 g Bacto-Tryptose, 3g Bacto-Soytone, 5 g NaCl, 2.5 g dipotassium phosphate, and 1 g dextrose per liter. This media served well for the J-series pseudomonads, but did not prove useful for the auxotrophic strains. Luria broth (Lennox, 1955), compounded of 10 g Bacto-Tryptone (Difco), 5 g yeast extract (Difco), 10 g NaCl, and 1 g dextrose per liter was adopted as the standard rich media.

Acetate and succinate minimal media. In cases where it was desirable to have a growth carbon source present, in addition to a phenolic inducing-compound, 1000 ppm of sodium succinate or sodium acetate (Eastman), adjusted with NaOH to pH7, was used in a basal mineral salts buffer base (BMS). Sodium succinate was prepared by dissolving 8 grams of anhydrous succinic acid (Mallinckrodt) in 80 ml of water, neutralizing with concentrated NaOh then diluting to 100 ml. Minimal media were then made by first autoclaving the BMS base, then adding the previously filter-sterilized acetate or succinate stock to the cooled buffer base.

Amino-acid supplementation. Supplementation with any required amino-acids was standardized at 40 ppm. Stock solutions of the L-amino-acids (HCl or free base; Sigma) were prepared by dissolving appropriate quantities in distilled water, to yield 10,000 ppm stock added at the rate of 4 ml per liter of culture broth. Stock solutions were filter-sterilized using disposable Nalgene 30 micron filters; proper amounts were added to the autoclaved media after it had cooled to 50^{0} C. or less.

Neomycin and Streptomycin antibiotic selective media. Neomycin sulphate (Sigma) as a 100 mg/ml stock was prepared with distilled water, filter sterilized as above, and stored

at 4°C. in the dark. Resistant strains used tolerated 400 ppm in any suitable growth media; sensitive strains showed no growth at that concentration. Streptomycin hydrochloride (Sigma) was prepared as a 100 mg/ml stock in distilled water, filter sterilized, and stored also at 4°C. in the dark; 10 ml/L of media yielded 1000 ppm standard concentration. The appropriate antibiotic concentrate was added to the autoclaved media base after it had cooled to 50°C. or less.

Phenolic media. Broth, plates and slants containing the cresols, phenol, or benzoate were prepared using 18,000 ppm stock solutions of the phenolic compounds. These stocks were not filter sterilized since repeated experiments had shown that contaminants do not survive such high concentrations. The brown glass storage containers, however, were sterilized prior to filling, and normal precautions were taken to prevent contaminants from surviving at the mouth of the bottles. Phenolics were added after the basal media had been autoclaved and had cooled to less than 50°C.

99%+ para-cresol, 99% meta-cresol, 99% ortho-cresol, and 98% phenol (all Aldrich Chemical Co.) were used as starting material; opened bottles were nitrogen-filled and recapped tightly. Stock solutions were also nitrogen-filled, tightly recapped, and stored at 4°C. in the dark between uses. Sodium benzoate was prepared from crystalline benzoic acid (Baker) by addition of the crystal to a dilute

NaOH solution containing sufficient NaOH to dissolve the acid. Any pH overshoot was adjusted with phosphoric acid, and a final dilution was made with distilled water to yield 18,000 ppm stock.

Interrupted Mating Procedure

Several techniques were evaluated while attempting to derive successful donor and recipient strains. The final method used to obtain cross-over times for the para-, meta-, ortho-cresol and phenol hydroxylase(s), and for the 2,3-oxygenase(s) in JPT3-4 was modelled after that of Jacob *et al.* (1960), with modifications introduced by Chakrabarty and Gunsalus (1969) and by Wu *et al.* (1972).

Following culture and preparation for mating (see *Culturing for mating experiments*, above), appropriate dilutions in 1% saline were taken from the two control flasks onto sets of nonselective and selective media plates for viability counts and contamination checks. For JPT3-4, the nonselective media were Luria agar, P900, M900, 0900, P100, M100, 0100. The selective media were P100-Neo, M100-Neo, 0100-Neo, and Luria-Neo (Neomycin sulphate at 400 ppm). For JPT8N, nonselective media were Luria, P900, P100, P100-Neo, and Luria-Neo; the selective media for

recombinants were M900, M100, M100-Neo, 0900, 0100, and 0100-Neo.

The contents of each pair of donor-recipient Erlenmeyer flasks were then added together, mixed by swirling, then allowed to stand undisturbed until sampled. Samples of 0.1 ml were drawn from a "mate mix" flask at predesignated times--normally at 15, 30, 60, 90, and 120 minutes into the mate--and diluted in 10^{-2} decrements onto recombinant-selective plates. Interruption in any donor-recipient mating was accomplished by intermittent, violent vortexing of the dilution tubes for 20 seconds during sample dilution, using a Vortex Jr. Mixer (Scientific Industries), prior to spreading a 0.1 ml sample of the final dilution onto a plate. Generally, on nonselective plates, a final concentration of 200 colonies per plate was desired. On selective media, that number represented either the number of donor or the number of recipient cells expected to survive; on recombinant-selective plates, dilutions placed a combined total of 10^4 to 4×10^4 cells on a plate. A final set of viability and contamination-check plates was made from each of the two control flasks at the end of the mating, also facilitating doubling-time quantification.

Plates were incubated at 28°C. Colony counts were taken on nonselective plates the following day; on selective plates in three to five days. Recombinant-selective media in JPT3-4 x JPT8N-80 mates were M100-Neo-supplemented BMS

agar. In JPT3-4 x PAS102N mates, plates were P100-Neo and M100-Neo BMS agar. Results were calculated as the number of exconjugants growing after 4 days on the recombinant-selective plates per donor cell present in the mate mix at T_o (initiation).

In mates where PAS102N acted as recipient, identical procedures were followed, except that the nonselective media were Luria and succinate-threonine (sodium succinate 1000 ppm, L-threonine 40 ppm); selective media for recipients were Luria-Neo or succinate-threonine-Neo (1000 ppm sodium succinate, 40 ppm L-threonine, and 400 ppm Neomycin sulphate, abbreviated ST-Neo); recombinant selective plates were P100-threonine-Neo and M100-threonine-Neo (P100T-Neo and M100T-Neo); all in a BMS agar base.

Single colonies detected on recombinant selective plates were streaked for isolation on appropriate selective media. Individual colonies were then picked off, streaked for growth in Luria agar slants for freeze-storage, and used to inoculate selective media slants which became the working cultures for oxygen probe, enzyme, and plasmid population studies on recombinants.

Chemicals

Amino acid supplements, antibiotics, TRIS (Sigma 7-9) buffer, PEG 6000, and agarose Type II were obtained from

Sigma and prepared as per published procedures referenced in the appropriate sections. The cresols, phenol, and catechols were purchased from Aldrich. Crystalline catechols were vacuum-sublimated and stored in brown phials under nitrogen at -40°C, until used; aqueous solutions were made fresh weekly and stored under nitrogen at 4°C.

Oxygen Electrode Studies

Oxygen consumption by whole cells. BMS buffer-washed, harvested cell pastes were resuspended in 50 ml BMS buffer at 0°C., and so maintained until used--usually within 30 minutes. Absorbances were typically 0.3 to 0.7, measured in a Nephelometer flask on a Coleman Jr. spectrophotometer at 600 nm. Four ml of these suspensions were placed in wells of a Yellow Springs Instrument Model 53 Oxygen Monitor and allowed to thermally equilibrate with the 30°C. water jacket while being bubbled with filtered air for two minutes. The wells were then sealed with the oxygen probe, and a one minute recording made of endogenous respiratory removal of oxygen from the vessel, using a Bausch and Lomb integrating recorder. Next, a 0.1 ml sample of a test substrate (a cresol isomer, phenol, sodium benzoate, a catechol, or protocatechuic acid, all at 3300 ppm) was injected, and changes in per cent oxygen in the test suspension recorded. Also on the test day, the barometric pressure (absolute or

corrected vs. relative, "station pressure," or uncorrected) and ambient temperature were recorded for use in the computer program that was developed to calculate and log the oxygen consumption of these suspensions as microliters of 0_2 consumed per hour per milligram of cells (dry weight).

Some .1 ml test substrate injections were done at 10,000 ppm; and another group done at 250 ppm. These high and low figures were found to yield less than maximal rates of oxygen utilization, with obvious inhibition at the high concentration for certain substrates. However, 3300 ppm in the injected sample yielded 83 ppm in the test cell, which was well within the concentration range promoting maximum utilization rates over the oxygen saturation range from 100% down to 2%. The computer program was designed to compensate for changes in the concentration of the injected test substrate, but most tests were made at the 3300 ppm concentration.

Oxygen consumption by enzyme extracts. In order to ascertain the absence of significant numbers of whole cells in enzyme preps (fully described in the following section), a number of oxygen electrode tests were made using 0.5 ml of enzyme extract in 3.5 ml BMS buffer, in place of the whole cell suspensions. Sonic disruption of the cellular membrane destroys the membrane-bound hydroxylases in JPT3-4, but

the 1,2- and 2,3-oxygenases remain active if no heat buildup is permitted in the sonication step. It was hypothesized that the absence of the hydroxylating activities in otherwise induced cell extracts could be taken as an indication of the completeness of cellular disruption. This assurance was required because, as later explained, the principal 2,3-oxygenase activities acting on the catechols could be located only in the debris pellet of these organisms; if the activity found was actually due to metabolism within remaining intact cells, then the rates observed might not be maximal, since membrane transport systems could be rate-limiting movement of substrate into whole cells.

Oxygen consumption and the presence of a yellow color in reaction mixtures were both used in the tracing experiments described in the following section.

Enzyme Studies

Isolation of 1,2- and 2,3-oxygenase activities. Initial enzyme studies at this laboratory by Hatcher (1977) on strain J1 had yielded positive oxygen electrode, spectrophotometric, and colorimetric evidence for a 2,3-oxygenase using the method of Bayly and Wigmore (1973) with modifications based on the work of Taniuchi *et al.*, (1962) and

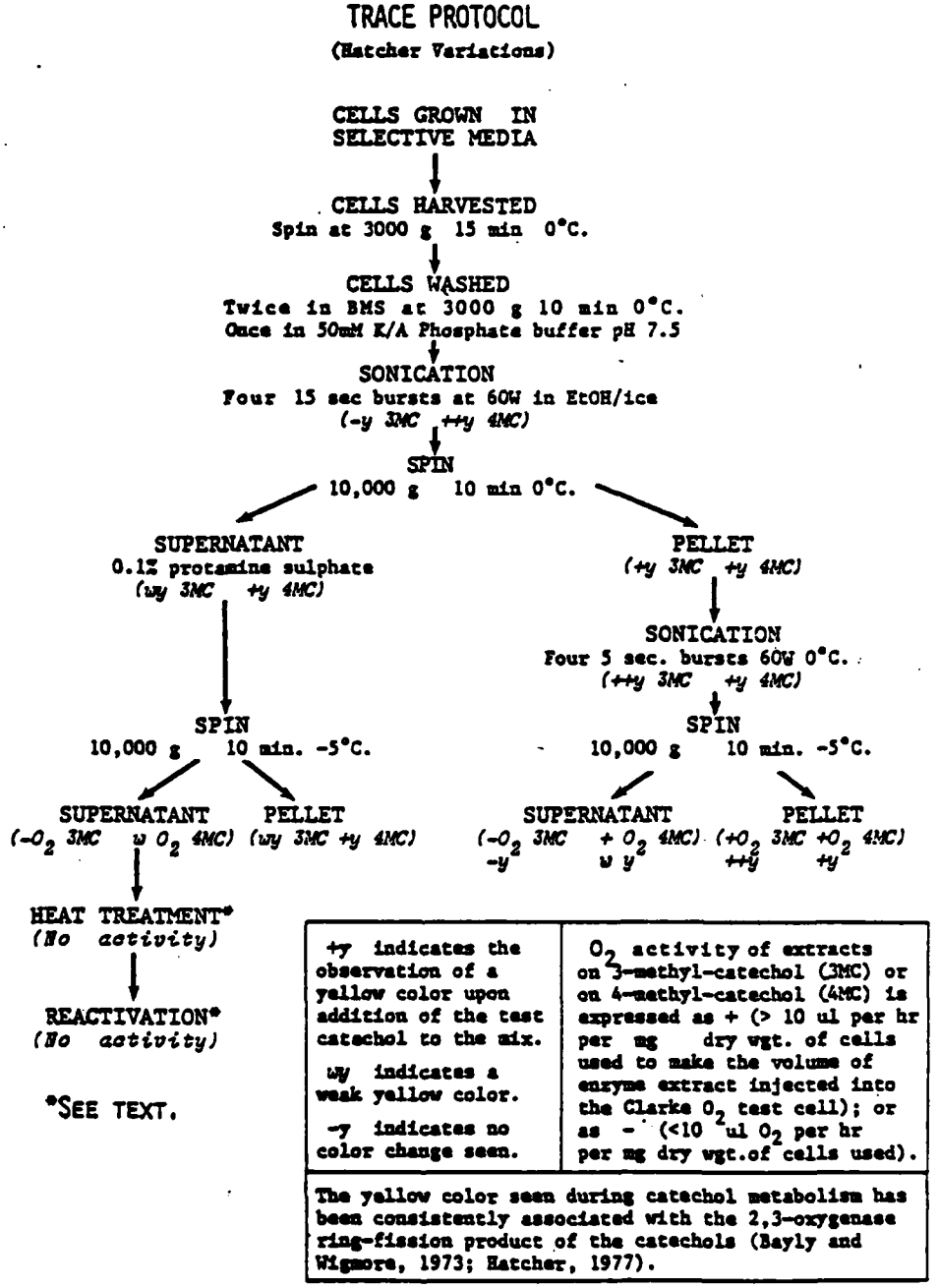
Hayaishi *et al.*, (1962). Hatcher's results showed a three- to seven-fold increase in absolute activity over those reported in the Bayly and Wigmore paper, a fact which we attributed to the use of Taniuchi's and Hayaishi's $NaBH_4$ "reactivation" step, plus the inclusion of 10% v/v anhydrous acetone as an anti-oxidant in the extracts. However, attempts to repeat these findings using the JPT3-4 organism were unsuccessful.

Olive and Burkholz (unpublished data), using the Hatcher procedure and cell extracts of JPT3-4 obtained by passing a dense cell suspension twice through a Gaulin hydraulic homogenizer (Type 15M8Ta) at 9000 psig, conducted spectrophotometric scans on a Cary Model 118C UV/VIS Recording Spectrophotometer of extract activity on the methyl-catechol derivatives of the cresols. They observed no peaks in the 400 nm to 350 nm region where "meta-fission" products were reported to occur (Bayly and Wigmore, 1973), but did detect some activity in the ultraviolet for catechol and 4-methyl-catechol (CAT and 4MCAT), indicative of 1,2-fissioning characteristic of the "ortho" pathway. When cell extracts were obtained by sonication (two, two-minute bursts using the microtip on a Lab-Line Ultratip Labsonic System (9100) operating at 20kc at 60W, with the system maintained at near 0°C. in an icewater bath), more 1,2OX activity was observed, but still no visible range products.

Since oxygen probe and colorimetric data continued to indicate the presence of a "meta-fission" pathway in cresol- and phenol-grown JPT3-4 cultures, it was clear that activity was not being recovered by the classic techniques. Accordingly, a set of tracing protocols were developed to track the 1,2- and 2,3-oxygenase activities to determine where they were being lost, first in the Hatcher technique, then in new procedures designed to avoid loss or destruction of the activities.

Preparation of cell extracts. Initial preparation of cell-free extract from JPT3-4 and JPT8 was accomplished using Hatcher's method (Fig. 3), with large (70g) batches of cells being processed through a Gaulin hydraulic homogenizer (Type 15M8Ta) at 9000 psig. (This facility was generously provided by the University of Texas Health Science Center, San Antonio, Texas.) Later a second method was introduced, wherein 3 to 7 gram batches of cells, prepared as described in *Culturing for enzyme extraction and activity experiments, p.14,* were resuspended in 5 ml 0.1uM KH_2PO_4 buffer in a small plastic test tube, and kept in a methanol-ice mixture. A 9108 microtip on a Lab-Line Ultratip Labsonic System 9100 ultrasonic disintegrator, operating at 60 W at 20kc, was used to administer four one-minute sonications, allowing a thirty second cooling-off period between

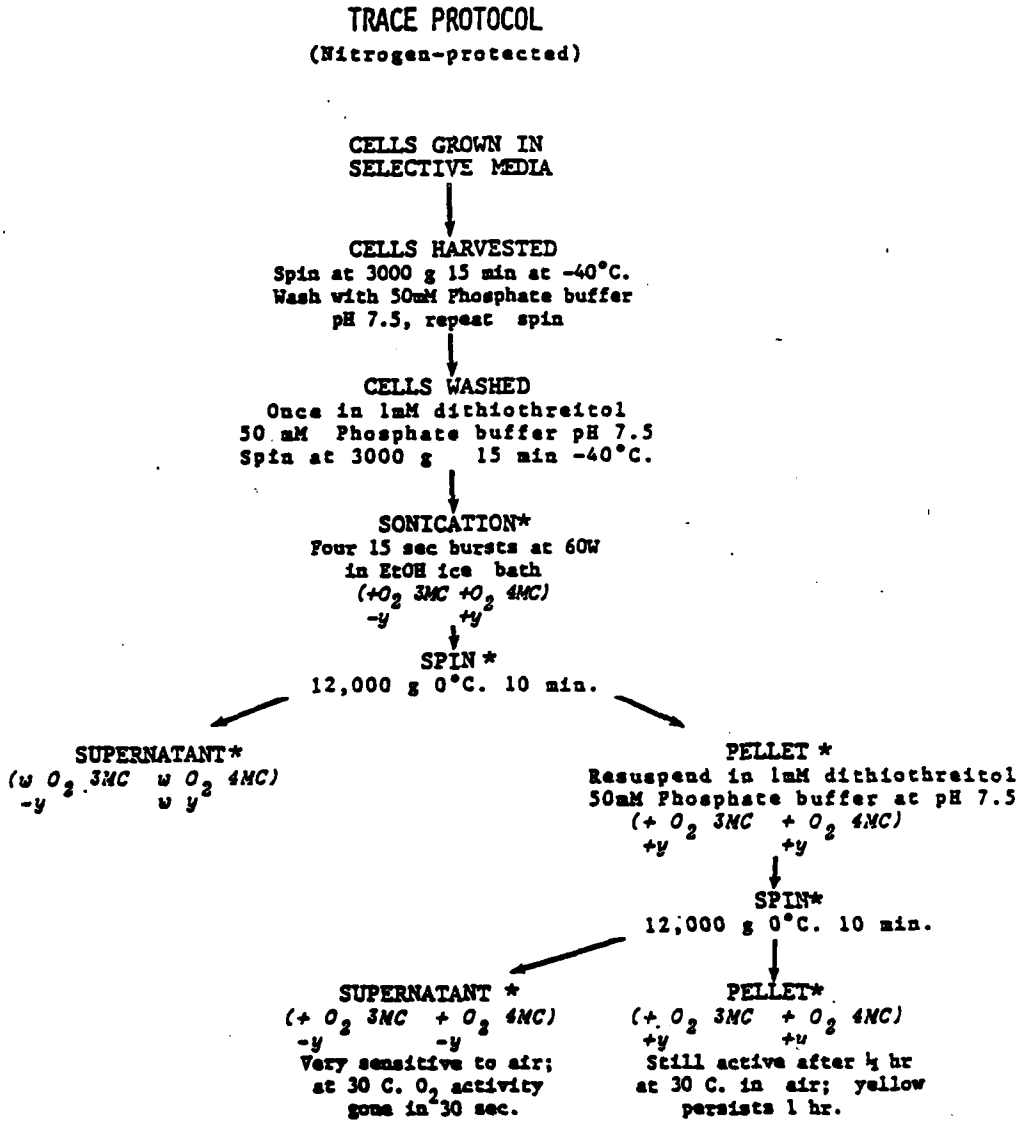
FIGURE 3


TRACE PROTOCOL
(Hatcher Variations)
CELLS GROWN IN SELECTIVE MEDIA
CELLS HARVESTED
Spin at 3000 g 15 min 0°C.
CELLS WASHED
Twice in BMS at 3000 g 10 min 0°C.
Once in 50mM K/A Phosphate buffer pH 7.5
SONICATION
Four 15 sec bursts at 60W in EtOH/ice
(-y 3MC +wy 4MC)
SPIN
10,000 g 10 min 0°C.
SUPERNATANT
0.1% protamine sulphate
(wy 3MC +y 4MC)
PELLET
(+y 3MC +y 4MC)
SONICATION
Four 5 sec. bursts 60W 0°C.
(++y 3MC +y 4MC)
SPIN
10,000 g 10 min. -5°C.
SPIN
10,000 g 10 min. -5°C.
SUPERNATANT
(-O2 3MC w O2 4MC)
PELLET
(wy 3MC +y 4MC)
SUPERNATANT
(-O2 3MC -y + O2 4MC) w y
PELLET
(+O2 3MC ++y +O2 4MC) +y
HEAT TREATMENT*
(No activity)
REACTIVATION*
(No activity)
*SEE TEXT.
+y indicates the observation of a yellow color upon addition of the test catechol to the mix.
wy indicates a weak yellow color.
-y indicates no color change seen.
O2 activity of extracts on 3-methyl-catechol (3MC) or on 4-methyl-catechol (4MC) is expressed as + (> 10 ul per hr per mg dry wgt. of cells used to make the volume of enzyme extract injected into the Clarke O2 test cell); or as - (<10 ul O2 per hr per mg dry wgt.of cells used).
The yellow color seen during catechol metabolism has been consistently associated with the 2,3-oxygenase ring-fission product of the catechols (Bayly and Wigmore, 1973; Hatcher, 1977).

exposures. These extracts were then treated by the Hatcher procedure prior to assay. If storage was necessary, extracts from both of the above procedures were frozen at -40°C. in 5 ml volumes in neoprene-seal screwcap test tubes until assayed.

After it was determined that none of these methods was preserving the 2,30X activities, a final preparative method was designed (Fig. 4). The resuspending buffer was 0.05M $KH_2PO_4 \cdot Na_2HPO_4$ (Hegeman, 1966) at pH 7.5 (Hopper and Taylor, 1975). Sonic disruption was accomplished under flowing nitrogen, with every precaution taken to maintain inert atmosphere conditions once sonication was begun. Eight 10-second bursts of 20kc at 60W, with 10-second cooling periods between, were applied to the suspensions, followed by centrifugation at 12,000 g at -10°C. in a Sorvall refrigerated centrifuge in small tubes covered with Parafilm. The supernatant was decanted under nitrogen into a small phial, then sealed and stored at -40°C. until assayed. The pellet fraction was resuspended in approximately 2 ml of the same buffer, using a 10-second burst of sonication to aid in the process, then again spun down. This time only the pellet was saved, resuspended in phosphate buffer, and stored in a brown, nitrogen-filled, diaphragm-capped bottle on methanol-ice. 2,30X assays were begun within 30 minutes.

FIGURE 4



* All steps handled under flowing nitrogen, or with tubes of extract materials sealed under nitrogen with parafilm during centrifugation and transfers, at 0 C. O_2 and spectrophotometer cuvettes of buffer solution were aerated and brought to room temperature prior to adding the test extracts.

Protein assays. Soluble proteins from the supernatant fraction of cell extracts were assayed according to standard instructions for the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, California). Protein in the pellet fraction of cell extracts was first finely suspended by brief sonication at 10W of the dye-enzyme mixture under nitrogen. All dye-enzyme mixtures were allowed a five minute incubation period at room temperature prior to reading absorbance at 595 nm on an Hitachi Perkin-Elmer Model 139 UV-VIS Spectrophotometer, using a plain dye blank and a series of bovine serum albumin standards. If necessary, extracts were diluted so that protein concentrations in the test cells fell within the 0.2 to 1.4 mg/ml range recommended for assay.

Enzyme assays. Catechol 2,3-oxygenase (2,3OX, EC 1.99.2.a) activities were measured by recording the rate of formation of the ring-fission products from the catechol substrates; catechol (CAT), 3-methyl-catechol (3MCAT), and 4-methyl-catechol (4MCAT) give visible peak absorbances at 375, 388, and 382 nm, respectively (Bayly *et al.*, 1966).

Catechol 1,2-oxygenase ("pyrocatechase," 1,2OX, EC 1.99:2.2) was assayed according to the method of Hegeman (1966) and Sistrom and Stanier (1954), modified principally in the preparation of the extract as detailed above. 1,2OX action on the catechols and protocatechuic

acid (PROCAT), with absorbance maxima near 260 nm, was recorded in separate tests. Reaction mixtures for both oxygenase assays contained 0.1 umole of a test substrate (except 3MCAT, where mixtures contained 1.0 umole in order to compensate for removal of ring-fission product by any "meta" pathway hydrolase) in 3.1 ml of Hegeman's phosphate buffer at pH 7.5, and 4.0 umole of EDTA, giving 4 ml total volume. (EDTA had been shown by Sistrom and Stanier (1954) to inhibit the subsequent lactonizing enzyme in the "ortho" pathway, thereby allowing accumulation of *cis-cis* muconate, the ring fission product of pyrocatechase, in the reaction cuvette.)

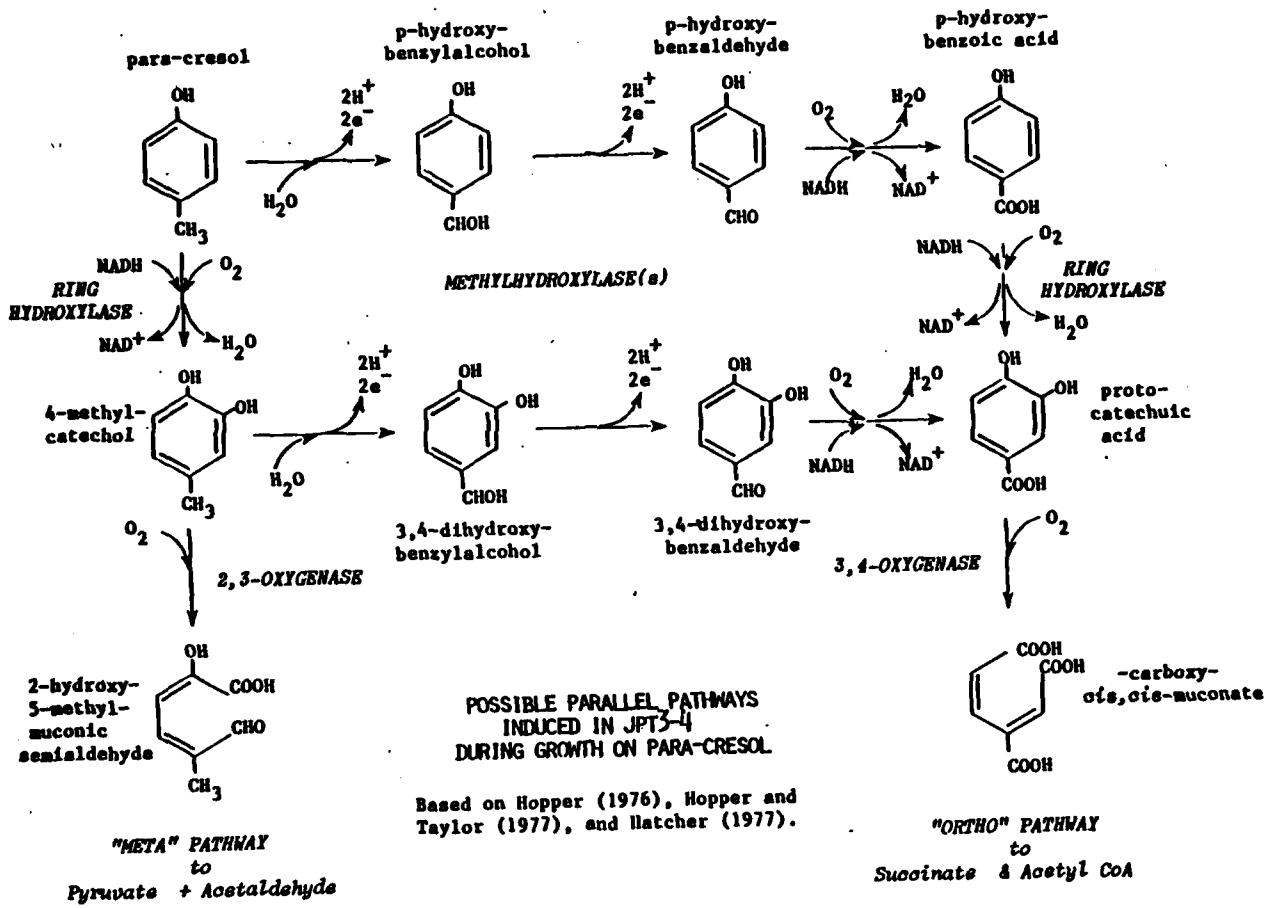
These components were mixed in 10 ml batches from cold substrate and EDTA, and room temperature buffer immediately prior to use to assure equal concentrations in both of the 10 mm path-length test and reference cuvettes. Reaction rate measurements were made at 23°C. on a Cary Model 118C UV-VIS Recording Spectrophotometer equipped with an automatic repetetive-scan feature. After obtaining a baseline, 0.05 ml of enzyme extract was drawn into a syringe from the sealed, nitrogen-filled brown storage phial kept on methanol-ice, injected into the test cuvette, and the cuvette inverted four times to assure mixing. For 2,3OX assays, repetetive scans covered from 400 to 350 nm; for 1,2OX activity, from 350 to 230 nm. Results are reported here as a range of characteristic product formation rates (+, ++, or +++), or the absence of such products (-).

The 3,4OX activity referred to throughout this paper is an "ortho" fissioning dioxygenase acting on protocatechuate (see Figure 6), splitting the diphenolic ring by an intradiol fission exactly analogous to the 1,2OX activity acting on the catechols (Ornston, 1966a). However, the protocatechuate 3,4OX is highly specific for its substrate (Fujisawa *et al.*, 1972), and does not act directly on catechol nor the methyl catechols.

Still other activities, not yet well-studied in the J-series organisms, have recently been detected by their characteristic set of UV peaks, generated by enzyme action on 4MCAT and CAT. It is not known at this time whether the activities seen represent other oxygenase products, or merely are buildups of metabolic products further down the induced "meta" fission pathway that is also functioning on 4MCAT and CAT under the conditions of the test (see RESULTS).

FIGURE 5

SUSPECTED METHYLHYDROXYLASE ACTIVITIES IN JPT3-4



POSSIBLE PARALLEL PATHWAYS INDUCED IN JPT3-4 DURING GROWTH ON PARA-CRESOL

Based on Hopper (1976), Hopper and Taylor (1977), and Hatcher (1977).

Plasmid DNA Extraction and Visualization

Efforts to extract extrachromosomal DNA were made for all experimental strains in Table 1 by several different methods, including two modifications of the Hirt precipitation (Guerry *et al.*, 1973; and Meyers *et al.*, 1976), the latter technique incorporating a Tris-saturated phenol extraction step. Still another modification was replacement of the phenol extraction by chloroform:isoamyl alcohol (24:1) extraction (M. P. Moyer, personal communication; modified after Timmis *et al*, 1974) within Meyers' technique. A final and entirely different approach utilizing PEG-precipitation (Humphreys *et al.*, 1974; and Hansen and Olsen, 1978) was eventually adopted as giving cleaner preps and best yields of small and large plasmids.

Visualization was accomplished using the techniques developed in Falkow's group (Falkow *et al.*, 1975; Meyers *et al.*, 1976), and based in part on work reported by Greene *et al.* (1974), with variations introduced to accomodate the different extraction techniques utilized. In the Hirt-based extractions (Hirt, 1967), the final step was always a resuspension of the ethanol-precipitated DNA in 100 to 150 ul TES buffer (50 mM NaCl, 50 mM EDTA, 30 mM TRIS, pH 8.0). This treatment of the DNA gave excellent results in vertical slab gel electrophoresis in our experience, but was reported to give less than optimum yields in large plasmid isolation. Later, when the Hansen and Olsen alkaline denaturation

technique was used, the PEG-precipitate was also resuspended in TES buffer; however, if the DNA were to be used for any other purpose, such as in endonuclease digests or for transformation, it first had to be cleared of PEG and any remaining SDS by ethanol precipitation followed by resuspension in 1 x SSC (0.15 M 1978). TES, SDS and PEG all interfered to varying extents with endonuclease digestion (Olive, personal observation).

After resuspension in TES, 5 to 20 µl of the DNA solution was mixed with 5 µl of E-dye (0.07% bromphenol blue, 7% SDS, and 33% glycerol in water), and loaded into wells in a 3 mm, 0.7% agarose gel (0.7% Sigma Type II agarose dissolved in E-buffer: 89 mM Tris base, 2.5 mM disodium EDTA, and 89 mM boric acid), in a Bio-Rad Laboratories Vertical Slab Gel Electrophoresis unit, Model 220. Using the E-buffer as electrolyte (Meyers *et al.*,1976) and a Gelman Model 38201 regulated voltage supply, samples were run for approximately 2.5 hrs at 100 mV and 30 mA on single gells. The gel was then removed and stained for 15 min in an aqueous 0.4 ppm solution of ethidium bromide, rinsed, and photographed through a Tiffen Photar 23A (orange) filter by fluorescent emissions stimulated by longwave UV, on Polaroid PN45 (positive-negative) film. All ethidium solutions, rinses, and stained gells were subjected to oxidation in concentrated Clorox oefore discarding.

RESULTS

Interrupted Matings

Mating results between the donor strain, JPT3-4, and various recipients are depicted in Table 2. Lack of success in some attempts, such as with PAO222-SR, were in most cases a time-dependent phenomenon; i.e., if the organisms were co-incubated long enough, some recombination was always detectable. This phenomenon might have been the result of restriction endonuclease systems in the recipient strains; such occurrences have been reported for PAO and in certain other *Pseudomonas* (Stanisich and Richmond, 1975). It was this interpretation of the results that led to abandonment of such strains as PAO222 as recipients, since gene cross-over in extremely long mates could not be strictly related to plasmid or chromosomal transfer times. However, recently another alternative explanation has surfaced: Studies with *Escherichia coli* mutants have demonstrated that an active DNA transport system (called a *symport* system) is required for mating competency in the recipient (Zoon *et al.*, 1976; López *et al.*, 1980), which is apparently inducible. The mate conditions used in this study were designed without regard for the induction requirements of such symport systems. The use of a late log phase shaking culture of recipients--one way to induce the DNA transferases--was based on empirical evidence that competency was greater in such cultures. Other

TABLE 2

INTERRUPTED MATING DATA

DONOR x RECIPIENT	INTERRUPT TIME (minutes)									
	5	15	30	60	75	90	105	120	180	240
JPT3-4 PAO222SR*	0	0	0	0	0	0	NT#	NT	NT	0
JPT3-4 JPT8N-80‡	0	0	0	0	0	0	.3	.5	NT	NT
JPT3-4 PAS102N ‡	NT	0	0	0.8	1	NT	NT	1.2	NT	NT

Data are given as number of exconjugants appearing on cresol media per 1000 donor cells originally inoculated into the mate media, under the stated conditions of mate.

* The designation SR signifies Streptomycin resistance.

‡ The designation N signifies Neomycin resistance.

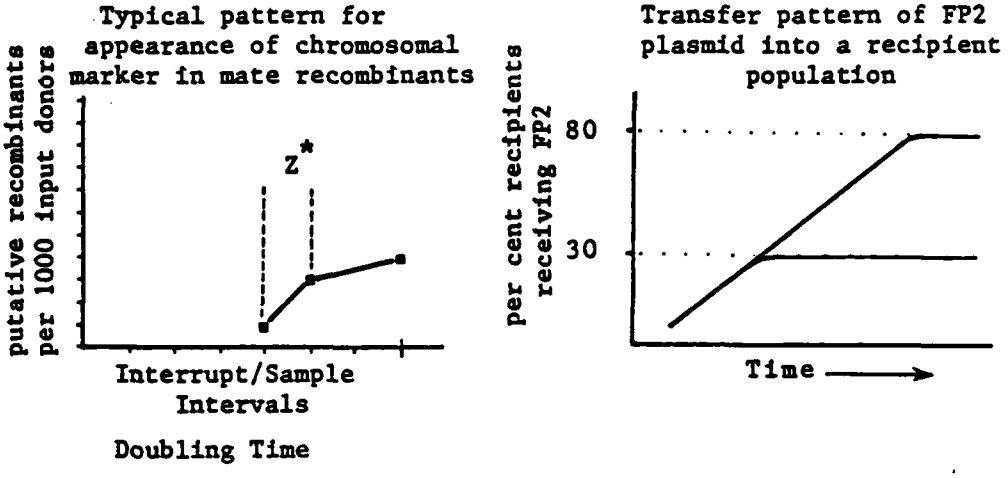
NT = Not Tested.

techniques may have given better stimulus to systems that might have better induced competency in *any* of the recipients tested.

Results from the successful matings are graphically depicted in Figures 6 and 7. Two trials for each successful recipient gave consistent data. Strain JPT8N-80 was a competent recipient derived from JPT8N, a strain which was, in turn, isolated during this study from a long-term co-incubation of the original J-series isolate, J1 (studied by Hatcher, 1977), and PTO13, a *Pseudomonas aeruginosa* carrying the FP2 sex factor and a *trp6* auxotrophic marker (see Table 3). JPT8N-80 was a prototrophic exconjugant having no detectable plasmid bands (except for the almost ubiquitous "front runner" described in the DISCUSSION, and labelled on Figures 19, 20, *et al.*, referred to as "linear DNA fragments" or "chromosomal fragments with an upper size limit" by several authors: Meyers *et al.*, 1976; Lopez-Alvarez *et al.*, 1980; White and Nester, 1980). JPT8N-80 demonstrated a $Pcre^{+}$ $MOcre^{-}\emptyset^{-}$ phenotype; *i.e.*, was able to utilize para-cresol (P) as a sole carbon source, but unable to efficiently utilize the meta- (M) or ortho- (O) cresol isomers or phenol (Ø or F in the tables and computer data printouts).

Exhaustive baseline data for JPT8N-80, obtained from oxygen electrode studies (*q.v.*) under a variety of induction conditions, showed this organism possessed a working hydroxylase for para-cresol, and inducible symport systems for

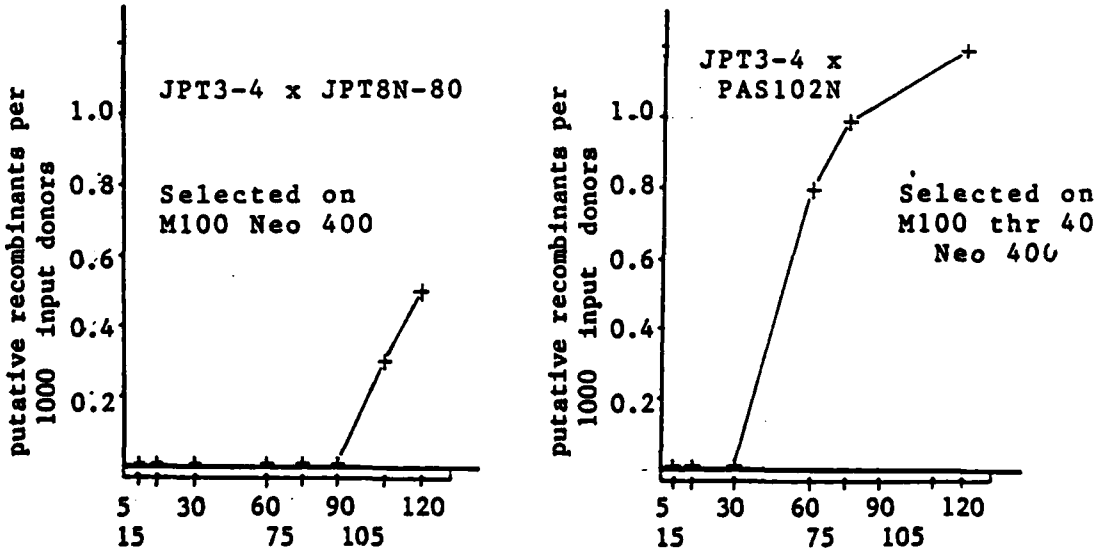
FIGURE 6



*The Z interval is complexly dependent upon synchronization of conjugation initiation, culture growth state, and other factors; post-Z slope reflects doubling rate of culture.

FIGURE 7

APPEARANCE OF CRE+ EXCONJUGANTS IN MATES WITH JPT3-4 AS DONOR



at least 4-methyl-catechol (4MCAT), catechol (CAT) and proto-catechuate (PCAT), and that the meta- and ortho-cresol isomers did in fact penetrate the cells without killing them. Thus, JPT8N-80 recombinants receiving only the crucial degradative genes--not necessarily any transport adjunct genes--ought to show up as positive growers on the selective media plates.

As can be seen from Figure 8, and Table 2, there appeared to be a definite and characteristic time-of-appearance for the capacity to grow on the meta-cresol isomer in the JPT3-4 x JPT8N-80 exconjugants, occurring at modest frequency (1 exconjugant per 3000 donor cells in the initial mate mix), at about 105 minutes into the mate. This pattern is normally indicative of a chromosomal linkage of critical degradative genes responsible for the selected phenotype. In these mates, the cross-over time for the *last critical gene locus* was T+105 min., corresponding to a position near the end of the JPT3-4 chromosome. (The doubling time for both JPT3-4 and JPT8N-80 controls under the given experimental conditions was 128 min., ± 10%

In crosses between JPT3-4 and PAS102N, PAS102N served as the female (recipient) strain. It, too, carried no detectable plasmids, but was auxotrophic for threonine (*thr102*), with an induced (and probably chromosomal, since no R-type plasmids could be isolated) Neomycin resistance. Recombinants from this mate pair were first detected at

T + 60 min., under conditions otherwise identical to those in the JPT3-4 x JPT8N-80 mate. This *last critical gene* crossing time is especially significant, and is covered in the DISCUSSION. Doubling time for PAS102N under mate conditions was 70 min. $\pm$ 10%, whereas JPT3-4 gave a nominal 115 min. $\pm$ 10%.

In the first crosses, JPT3-4 x JPT8N-80 exconjugants were picked from M100-Neo selection plates, where they had appeared as both small and large colonies after five days' incubation. A total of eight colonies were selected for purification (isolation-streaking on selective agar of individual colonies), followed by electrophoretic analysis of plasmid DNA populations. From the two types of exconjugants so identified--those possessing and those lacking the characteristic JPT3-4 plasmid band--two isolates were studied in oxygen electrode induction experiments, and their cell extracts used in spectrophotometric enzyme assays, as reported below.

In the JPT3-4 x PAS102N mates, putative recombinants appeared as both small and large colonies on the M100-thr-Neo selection plates after four days' incubation. Following purification, electrophoresis also demonstrated a division of the "recombinant" population, but into three rather than two distinct groups: (1) those large colonies which possessed a typical JPT3-4 plasmid band; (2) those small colonies also demonstrating the plasmid; and (3) those small colonies

which did not yield evidence of harbouring the plasmid (see Table 3 and Figure 8). Interestingly, all of the isolates from the recombinant selective plates carried the *thr*⁻ genotype characteristic of the recipient strain, PAS102N. This fact clinched the identification of the exconjugants as recombinants, and simultaneously indicated that the *thr* locos must come relatively late on the JPT3-4 donor's chromosome.

Four exconjugants representing the three groups were chosen for oxygen electrode analyses, supplemented by enzyme extract spectrophotometric tests; these were designated strains J3-102A, B. C, and D (see Figures 8, 9, 10, 11, 12, and 13).

Whole Cell and Enzyme Extraction Studies

Tabulated data from induction tests on both parent strains and putative recombinants are to be found in Figures 8 through 13 which provide graphic "induction fingerprints" for roughly comparing response sets in the oxygen electrode studies. The values are extracted from the Computer Data Tables. A compressed tabulation of qualitative results is available in Table 3.

Cresol and phenol hydroxylases (HX activities). Oxygen electrode results for whole cells fed cresols or phenol actually reflect netabolism of both the primary substrate and its catechol metabolite. Sala-Trepat *et al.* (1972)

TABLE 3

RELATIONSHIP BETWEEN PRESENCE OF THE FP2 PLASMID AND POSSESSION OF INDUCIBLE PHENOLIC HYDROXYLASE AND OXYGENASE ACTIVITIES IN DONOR, RECIPIENTS, AND EXCONJUGANTS

Organism	Source	Group¶	Plasmid	PHX	MOØHX	1,2OX 3,4OX	2,3OX
JPT3-4	J1xPT013	I	+	+	+	+	+
JPT8N-80	" "	II	-	+	-	+	-
PAS102N	(Walker)		-	w	w	w	w
J3-8R1-A	JPT3-4xJPT8N*	I	+	+	+	NT	NT
J3-8R1-B	" "	I	+	+	+	NT	NT
J3-8R1-C	" "	II	-	+	-	NT	NT
J3-8R1-D	" "	II	-	+	-	NT	NT
J3-8R1-E	" "	III	+	+	+	+	+
J3-8R1-F	" "	II	-	+	-	+	-
J3-102-A	JPT3-4xPAS102N	III	+	+	+	+	+
J3-102-B	" "	IV	+	+	w	+	+
J3-102-C	" "	III	+	+	+	+	+
J3-102-D	" "	II	-	+	-	+	-
J3-102-E	" "	NA	+	NT	+	NT	NT
J3-102-F	" "	NA	+	NT	+	NT	NT
J3-102-G	" "	NA	+	NT	w	NT	NT
J3-102-H	" "	NA	+	NT	w	NT	NT

N following organism designation indicates Neomycin resistance.

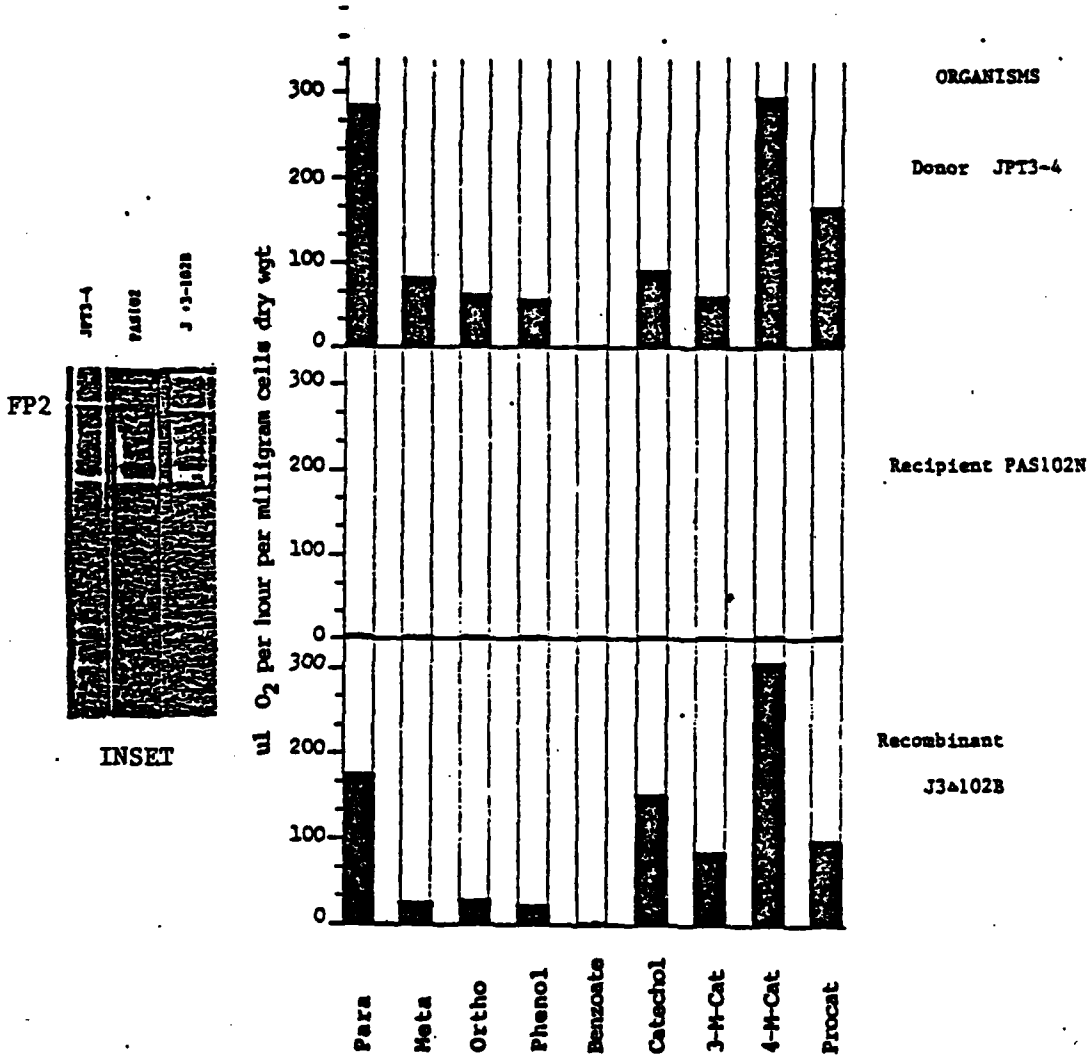
NA = Not Assignable
NT = Not Tested
*T+105 min

¶ Group designations are explained in text.

PHX = para-cresol hydroxylase.
MOØHX = meta-, ortho-cresol and phenol hydroxylase activities.
1,2/3,4OX = "ortho-fissioning" activities.
2,3OX = "meta-fissioning" activities.
+ = above 35 μmoles O_2/hr·mg cells
w = 5≤ w ≤ 35 μmoles O_2/hr·mg cells
- = <5 μmoles O_2/hr·mg cells

FIGURE 8

OXYGEN ELECTRODE INDUCTION "FINGERPRINTS"



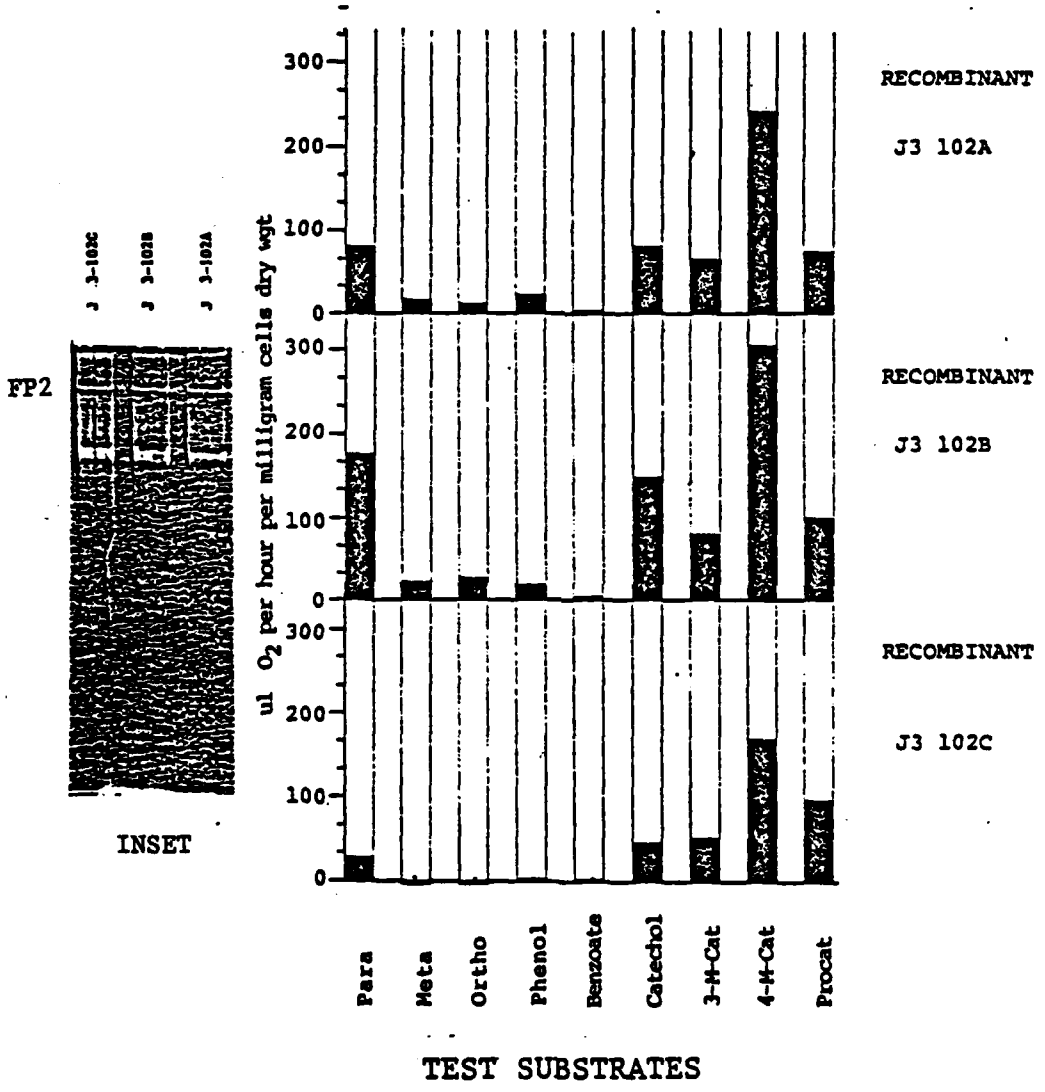
INDUCER: para-cresol
ALTERNATE CARBON SOURCE:

for JPT3-4:	none	*for PAS102N:*	1000 ppm succinate
for J3-102B:	40 ppm threonine supplement only		40 ppm threonine

Agarose gel electrophoresis run in inset shows the FP-type plasmid band borne by donor JPT3-4 and exconjugant J3-102B, while recipient PAS102N carries no such band. PAS102N was grown initially on succinate in this experiment due to the high toxicity of cresols to the recipient.

FIGURE 9

OXYGEN ELECTRODE INDUCTION "FINGERPRINTS"

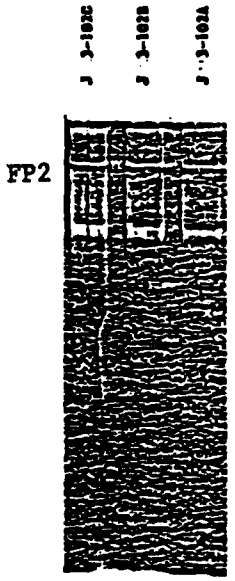


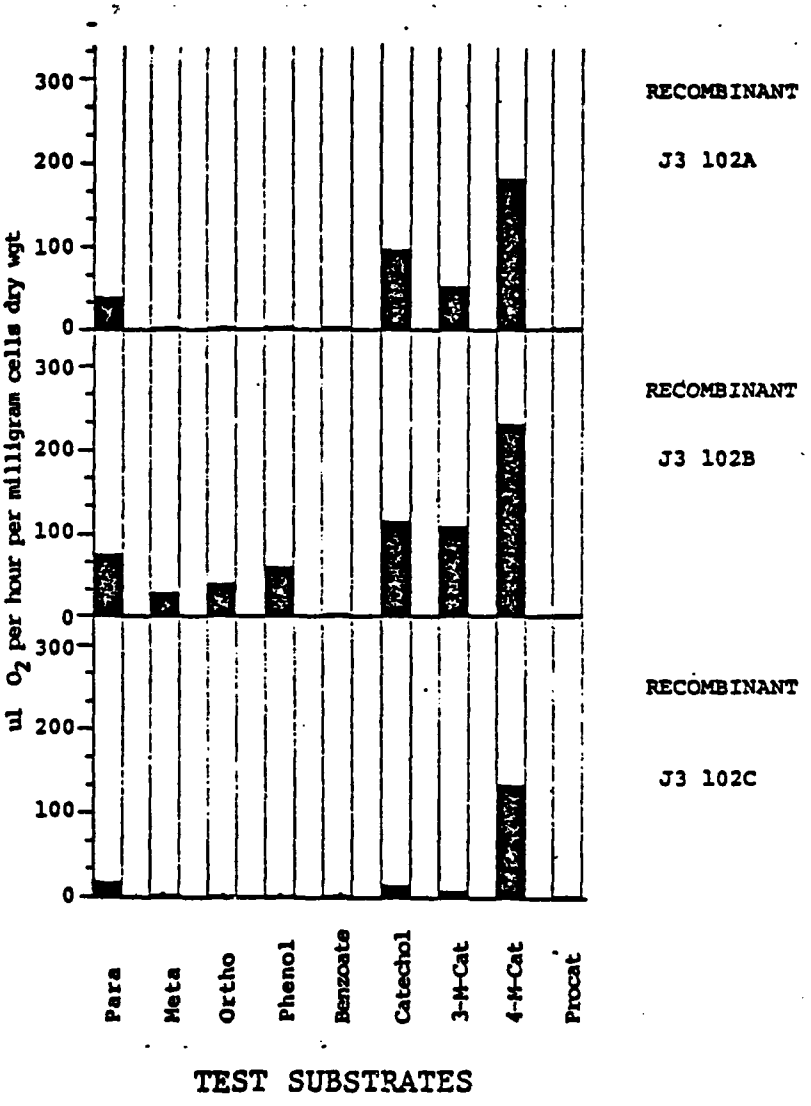
INDUCER: 250 ppm para-cresol
ALTERNATE CARBON SOURCE: 1000 ppm succinate
40 ppm threonine supplement

Agarose gel electrophoresis run in inset shows the FP-type plasmid present in each of these JPT3-4 x PAS102N exconjugants.

FIGURE 10

OXYGEN ELECTRODE INDUCTION "FINGERPRINTS"



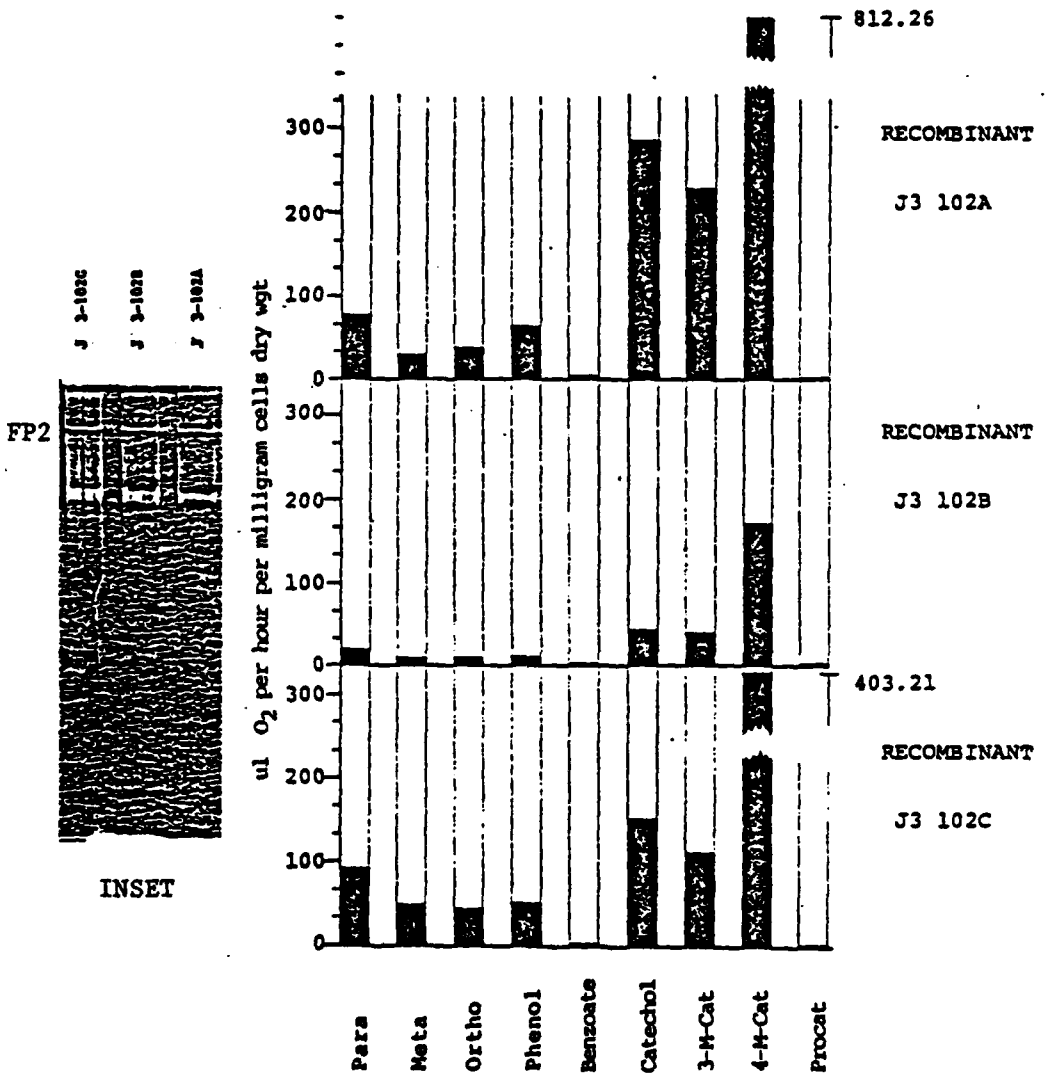


INDUCER: 250 ppm meta-cresol
ALTERNATE CARBON SOURCE: 1000 ppm succinate
40 ppm threonine supplement

Agarose gel electrophoresis run in inset shows the FP-type plasmid present in each of these JPT3-4 x PAS102N exconjugants. Note the poorly induced hydroxylase activities, indicating that some aspects of induction control may be determined by separate gene groupings.

FIGURE 11

OXYGEN ELECTRODE INDUCTION "FINGERPRINTS"

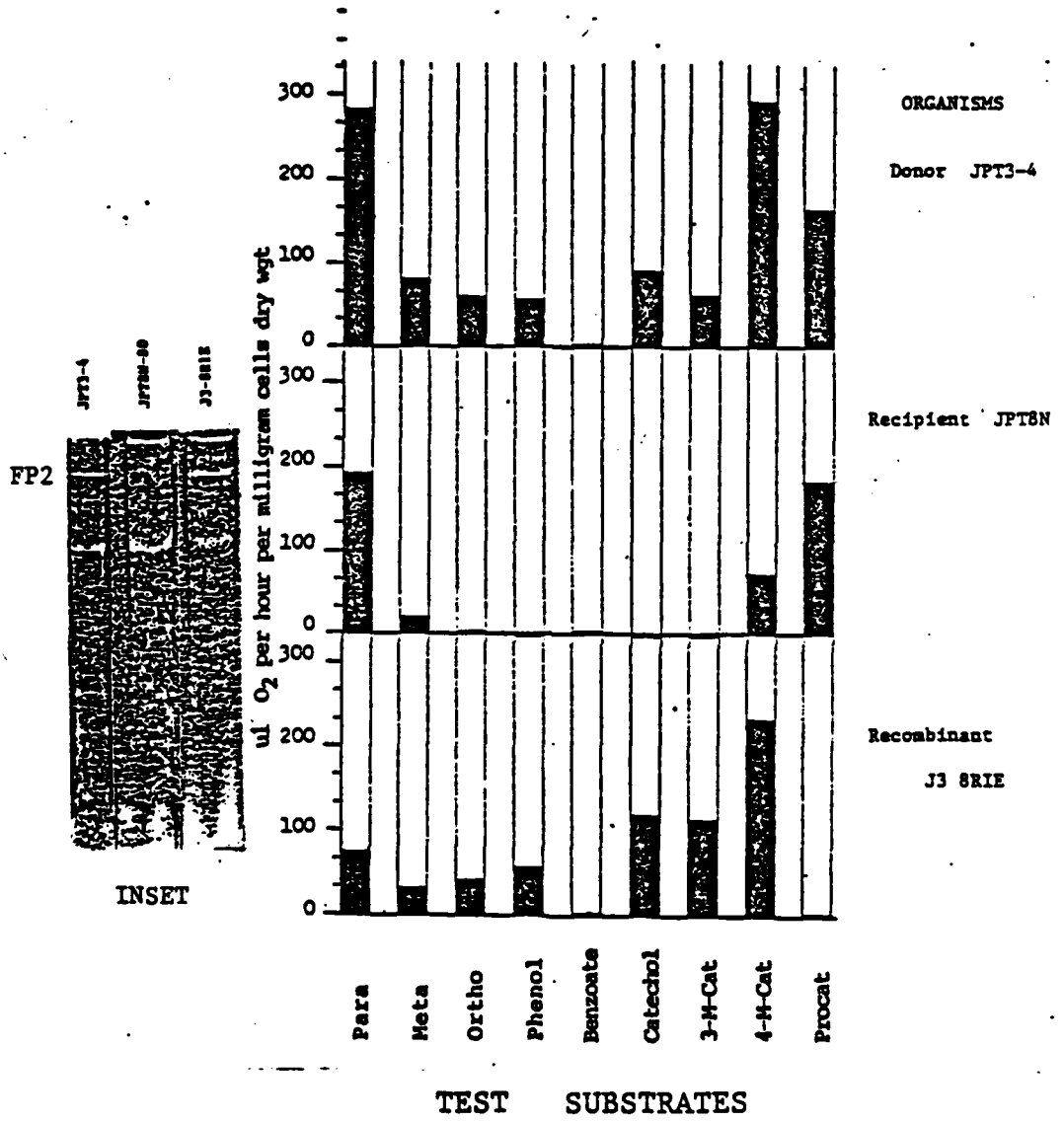


INDUCER: 250 ppm phenol
ALTERNATE CARBON SOURCE: 1000 ppm succinate
40 ppm threonine supplement

Electrophoresis run in inset shows FP-type plasmid present in these three JPT3-4 x PAS102N exconjugants. Note range of expression of the oxygenases, and compare to the performance of parental donor JPT3-4 in Figure .

FIGURE 12

OXYGEN ELECTRODE INDUCTION "FINGERPRINTS"

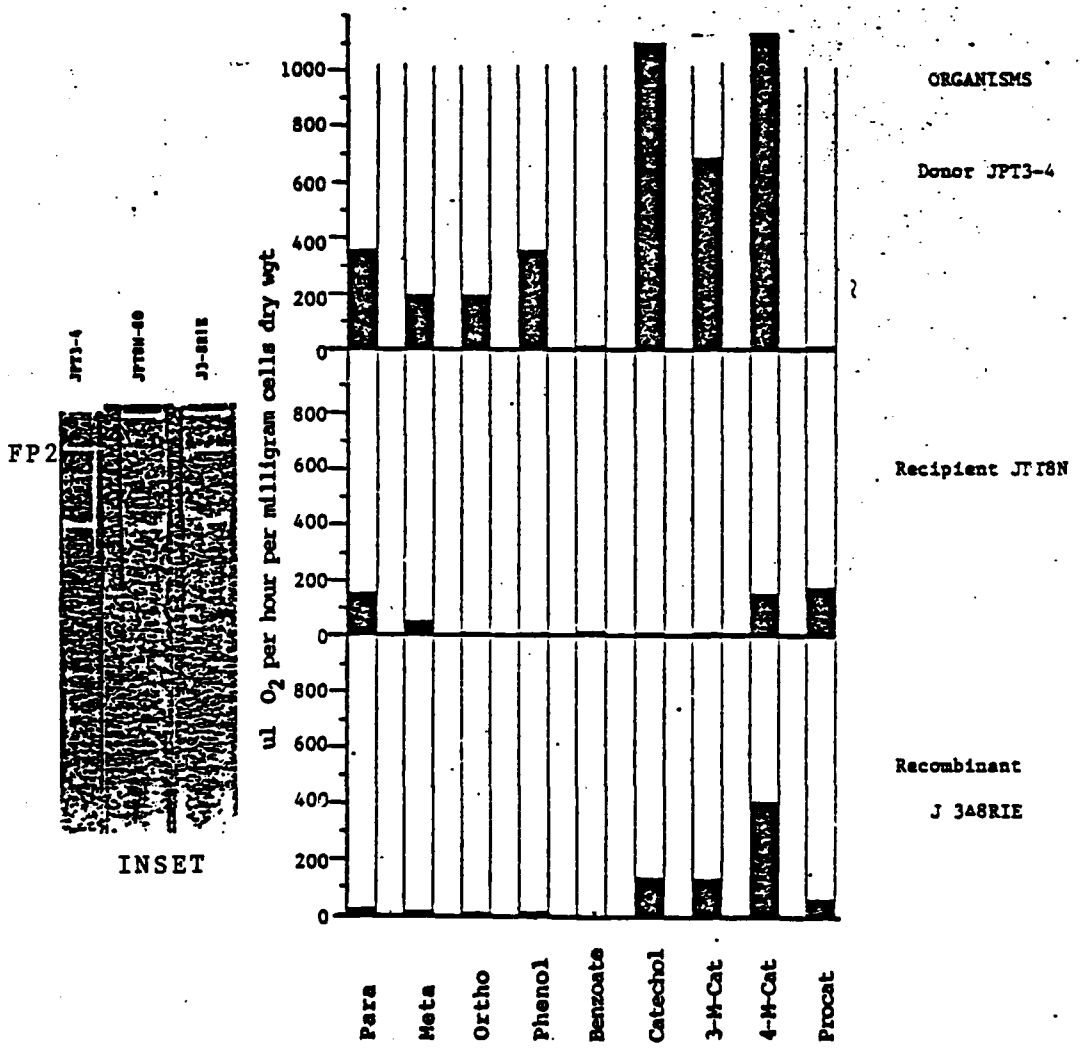


INDUCER: para cresol
ALTERNATE CARBON SOURCE: none

Agarose gel electrophoresis run in inset shows the FP-type plasmid band present in the donor, JPT3-4, and exconjugant, J3-8R1E; recipient JPT8N-80 shows no FP band.
Note that presence of an induced set of hydroxylases and the meta-fissioning oxygenases seems to interfere with normal induction of the PROCAT activity in the recombinant.

FIGURE 13

OXYGEN ELECTRODE INDUCTION "FINGERPRINTS"



INDUCERS: para-cresol and ortho-cresol
ALTERNATE CARBON SOURCE: none

Agarose gel electrophoresis run in inset shows the FP-type plasmid band present in the donor, JPT3-4, and exconjugant, J3-8R1E; recipient JPT8N-80 shows no FP band.

Compare the induction pattern in the plasmid-bearing J3-8R1E with that induced in the donor and recipient. Ortho-cresol, although weakly inducing a set of oxygenase activities for the catechols in the exconjugant, appears to retain its inhibitory effect on the host cell's machinery.

had found the HX step to be rate-limiting in several strains of *Ps. putida,* and it was clear from a comparison of cresol *versus* catechol rates in this study that the hydroxylation reactions were either rate-limiting themselves, or were following rate-limiting symport or antiport systems.

JPT3-4 baseline data (Table 4) show that the organism oxidized all nine of the standard test compounds via inducible activities. Unfortunately the specificity of the various inducible activities is not at all clear-cut. Para-cresol induced cultures showed a strong predilection for para-cresol, but operated with lower efficiency on meta- and ortho-cresol and phenol. Meta-cresol induced cultures showed a variable pattern, favoring para-cresol except when acetate had been included with para-cresol in the growth media, in which case the oxidizing activities were about equal for the three isomers and phenol. Ortho-cresol and phenol proved the best inducers of both hydroxylating and ring-fissioning activities, eliciting strong para-cresol and phenol oxidation, and only slightly less rapid oxidation of meta- and ortho-cresol. Benzoate was oxidized only when benzoate had served as an inducer, although the combination of benzoate and para-cresol seemed to potentiate the induction of benzoate oxidase.

A brief series of tests made on JPT3-4, reported in Figure 14, extend these results to cultures induced on PROCAT, parahydroxybenzoic acid (PHBCooH), parahydroxybenz-

TABLE 4

BASELINE INDUCTION DATA

Oxygen utilization in ul O_2 per hour per mg of cells, dry weight, for induced cultures of donor and recipient organisms. Growth substrates were also the inducing substrates for JPT3-4. For JPT8N-80, para-cresol, benzoate, and acetate were growth substrates as well as inducers; other phenolics were inducers only. Initial concentration of phenolics was 500 ppm for these two organisms. PAS102N was grown 24 hrs on 1000 ppm succinate with 40 ppm threonine, then induced two hours prior to harvest on 250 ppm of the phenolic inducer substrate. (-) indicates inhibition of endogenous respiration by test substrate.

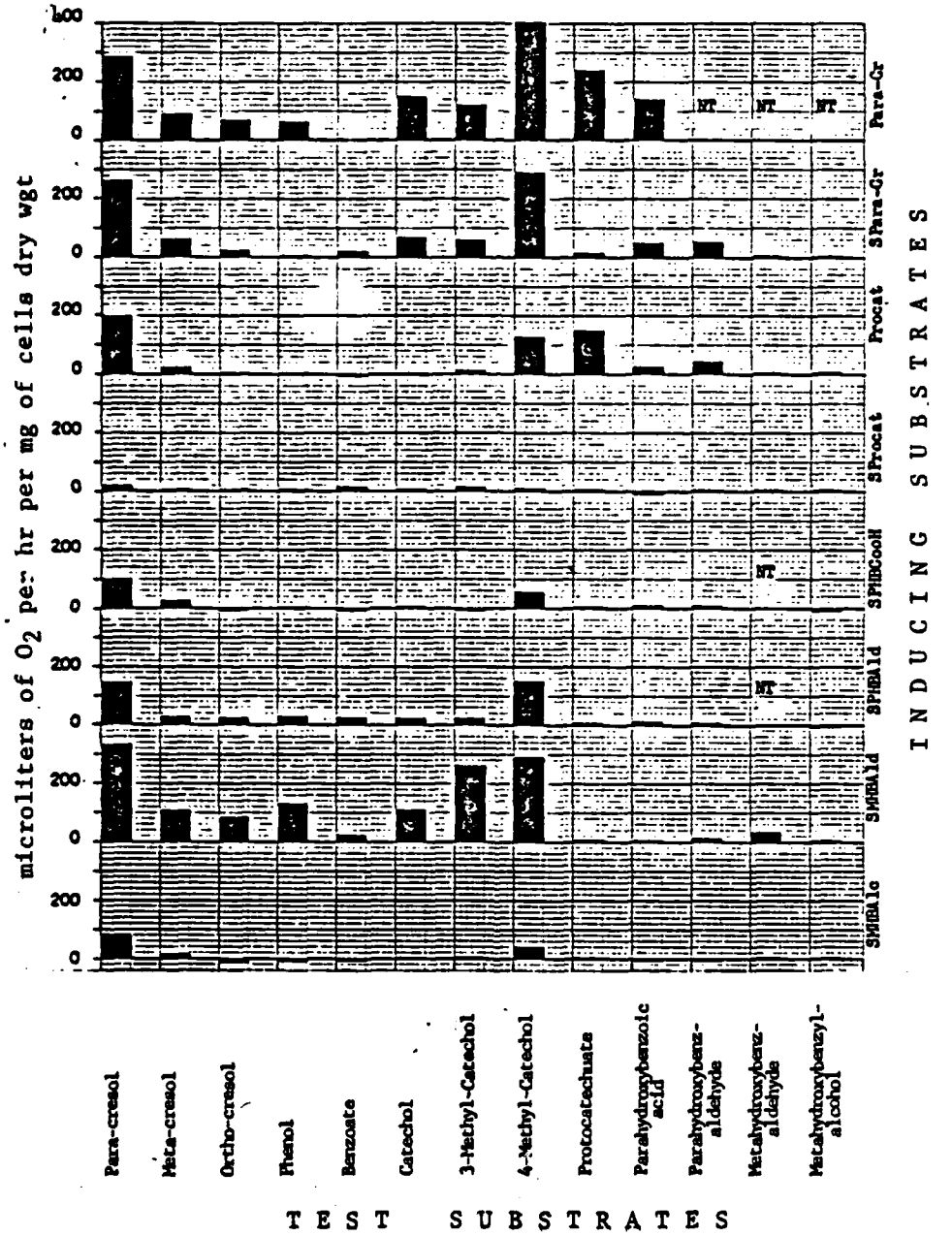
		TEST SUBSTRATES								
	INDUCER	PARA	META	ORTHO	PHENOL	BENZ	CAT	3MCAT	4MCAT	PCAT
JPT3-4	Para-cresol	279	87	57	53	2	90	63	285	166
	Para-cresol/Acetate	223	63	34	43	-	29	55	113	19
	Meta-cresol	113	67	67	84	0	377	277	579	25
	Meta-cresol/Acetate	182	186	200	159	-	589	520	759	34
	Ortho-cresol	349	186	181	352	3	1073	642	1095	2
	Phenol	333	275	294	428	-	727	791	1297	-
	Phenol/Acetate	66	50	NT	81	3	103	112	293	1
	Phenol/Para-cresol	52	-	-	-	-	50	42	229	128
	Phenol/Benzoate	98	68	NT	72	67	649	610	609	-
	Benzoate/Acetate	102	23	NT	3	97	182	48	156	2
	Benzoate/Para-cresol	103	70	NT	6	301	196	186	264	130
	Acetate	83	25	18	-	-	-	-	70	-
JPT8N-80	Para-cresol	185	20	2	-	1	-	-	70	173
	Para-cresol/Acetate	166	49	23	-	-	30	25	70	56
	Meta-cresol/Acetate	21	23	11	12	12	-	-	23	16
	Meta-cresol/Benzoate	133	38	5	7	44	183	27	121	22
	Ortho-cresol/Acetate	2	10	4	0	0	1	0	4	0
	Ortho-cresol/Para-cresol	165	58	7	3	2	3	6	148	184
	Phenol/Para-cresol	158	79	0	1	3	24	9	90	100
	Phenol/Benzoate	64	13	3	-	57	286	35	257	4
	Benzoate	100	19	10	0	140	380	48	233	14
	Benzoate/Acetate	75	37	-	-	22	20	-	44	13
	Benzoate/Para-cresol	145	15	3	5	26	57	9	76	72
	Benzoate/Meta-cresol	133	39	5	7	44	183	27	121	22
	Acetate	55	33	-	22	26	-	-	33	26
PAS102N	Para-cresol*	3	-	4	-	1	2	0	5	1
	Meta-cresol*	4	4	1	12	0	0	2	4	9
	Ortho-cresol*	-	-	3	2	2	0	0	0	3
	Phenol*	5	3	-	2	0	-	3	2	2

* ALL PAS102Ns were grown 24 hrs on 1000 ppm sodium succinate with 40 ppm threonine, then induced two hours prior to harvest on 250 ppm of the phenolic inducer substrate.

(-) indicates inhibition of endogenous respiration by the test substrate.

FIGURE 14

EVIDENCE FOR A PARA-CRESOL/PROCAT PATHWAY IN JPT3-4



Examination of this set of induction "fingerprints," giving oxygen utilization during test substrate metabolism for JPT3-4 cultures induced on intermediates in the PARA-CRESOL to PROTO-CATECHUATE pathway, shows the evidence for this pathway in JPT3-4. Note that metahydroxybenzaldehyde (MHBAld) is a strong gratuitous inducer of the "meta-fission" and PROCAT pathways.

aldehyde (PHBAld), metahydroxybenzaldehyde (MHBAld) , and metahydroxybenzylalcohol (MHBAlc). Since JPT3-4 would not grow on the benzoic acids or aldehydes as sole carbon sources fast enough for use, succinate was included as a growth substrate. Succinate was also included with a para-cresol culture and a protocatechuate (PROCAT) culture to reveal any interfering effects with induction. Figure 14 does show some reduction in induced activities when the alternate carbon source is present, compared to when it is absent, with the phenol hydroxylase and PROCAT oxygenase being the most strongly affected. Interestingly, PROCAT as an inducer elicits activities for only para-cresol, 4MCAT, and itself, with moderately low activities for parahydroxy-benzoic acid and parahydroxybenzaldehyde. All of these substrates have been shown to be in a common *Pseudomonas* catabolic scheme for the degradation of para-cresol, through the benzyl alcohol, benzaldehyde, and benzoic acid intermediates to protocatechuate, followed by 3,4-oxidation and subsequent reduction to β-ketoadipate (Dagley and Patel, 1957; Bayly *et al.*, 1966; Ornston and Stanier, 1966; Chapman and Hopper, 1968; Hewetson *et al.*, 1978). An anomalous and pronounced induction of all the hydroxylase and oxygenase activities for all of the major test substrates except PROCAT is elicited by inclusion of metahydroxybenz-aldehyde in the growth media.

JPT8N-80 gave a very different picture. Extracted baseline data in Table shows a para-cresol-specific oxidizing activity, induced efficiently by para-cresol and only somewhat less strongly by benzoate. In these cases, meta-cresol was oxidized at low to moderate rates, always remaining a roughly constant fraction of the para-cresol activity. Benzoate oxidase was strongly induced by growth on benzoate, but inclusion of other phenolics either greatly decreased or obliterated its induction. JPT8N-80 would not grow on meta- nor ortho-cresol, nor phenol as sole carbon sources; combinations of these phenolics with acetate, or with para-cresol or benzoate, yielded no oxidation activities for them, rather, the magnitude of normally induced activities were reduced, with a single exception: the combination of meta-cresol and benzoate did yield increased oxidation of para- and meta-cresol, when compared to benzoate induction alone.

PAS102N showed virtually no activity whatsoever on phenolic substrates, regardless of inducing conditions. The endogenous respiration rates of the various cultures were often inhibited by the presence of as little as 83 ppm of the test substrates, perhaps indicating the absence of ancillary systems that in, for instance, JPT8N, provide some measure of protection to cellular machinery from the toxic effects of the phenolics, even when no degradative enzymes have been induced in the organism.

Concerning apparent hydroxylase activities in putative recombinants, in experiment sets run on exconjugants from JPT3-4 x JPT8N-80 and JPT3-4 x PAS102N, oxidation rate differences were relatively clear-cut. Both combinations yielded strain variants expressing a range of donor hydroxylase activities. Because of the close tie-in to their expression of oxygenase activities, these data are reported in the following section.

Catechol 1,2-, 2,3-, and 3,4-oxygenases (1,2OX, 2,3OX, and 3,4OX). Washed JPT3-4 cell suspensions, which had been grown with para-cresol as sole carbon source, rapidly oxidized catechol (CAT), and 3- and 4-methyl-catechol (3MCAT and 4MCAT), as well as protocatechuate (PROCAT). Strong yellow colors in the reaction vessels for CAT and 4MCAT were indicative of "meta" ring-fission products (Hopper and Taylor, 1975). The absence of color during rapid 3MCAT oxidation was later determined to be due to the efficient metabolism of the colored ring-fission product (see enzyme data below, and also Ribbons, 1966). PROCAT oxygenase induction was severly depressed when acetate was included along with para-cresol in the inducing growth media; nor was PROCAT metabolized by whole JPT3-4 cells induced on meta- or ortho-cresol, phenol, or benzoate, although the presence of these latter phenolics with para-cresol in the growth media did not prevent induction of oxygenase activity.

The oxygen uptake rate for 4MCAT was much higher than that of CAT or 3MCAT in para-induced cells, a pattern repeated for every inducer except the combinations of benzoate/phenol and benzoate/acetate, for which CAT and 4MCAT activities were more nearly equal. Meta-cresol gave an accentuated "para" pattern for the catechols, heavily inducing 4MCAT activity, but elicited only a low PROCAT activity. Acetate as an alternate carbon source with meta-cresol stimulated the catechol oxygenase activities but did not significantly alter their apparent specificities. Phenol gave an even greater intensification of the oxygenases, again maintaining the "para" pattern favoring 4MCAT, but with truly incredible rates (Table 4) (compare Ornston, 1966; Ribbons, 1966; Feist and Hegeman, 1969; Bayly and Wigmore, 1973; and Hatcher, 1977). Benzoate, on the other hand, induced CAT and 4MCAT activities about equally, having some four times the rate for 3MCAT; in combination with phenol, it yielded high, equal activities for the catechols. Only when para-cresol was included with benzoate was any PROCAT activity observed. In Figure 14, oxygenase activities on the catechols are also induced by para- and metahydroxybenzaldehyde. PROCAT activity was induced only by PROCAT, and, of course, para-cresol. Oxygen uptake when para-cresol, PROCAT and metahydroxybenzaldehyde induced cells are presented the hydroxybenzoicacid and aldehyde substrates is probably hydroxylation activity (Hopper, 1976).

The recipient organism, JPT8N-80, typed, like JPT3-4, by the Ft. Sam Houston Army Pathology Laboratory as *Pseudomonas aeruginosa*, exhibited a *Pcre*$^{+}$ *MOcre*$^{-}$ $\emptyset^{-}$ phenotype which appeared to be due to a total lack of hydroxylating activities specific for meta- and ortho-cresol and phenol, and a lack of inducible 2,3-oxygenase activities. The oxidation of CAT and 4MCAT, and moderately low use of 3MCAT in some cases, was shown by enzyme assay methods to be due to 1,2-oxygenases (see *Enzyme studies results*, below). A good PROCAT oxidative activity was observable when JPT8N-80 was induced on para-cresol, para-cresol with ortho-cresol, para-cresol with phenol, and para-cresol with benzoate (Table 4). Growth with acetate alone gave only low and moderately low levels of para- and meta-cresol hydroxylase, phenol hydroxylase and benzoate oxidase activity, 4MCAT and PROCAT oxygenase activities.

PAS102N demonstrated essentially no induced oxygenases. When grown on low-concentration phenolic media, even when supplied with 40 ppm threonine (enough to sustain growth when succinate was the carbon source for this auxotrophic strain), this recipient strain exhibited a marked concentration sensitivity to the phenols (see Table 5). A test cross of PAS102N x JPT8N-80 was made in order to check for the presence of any donatable genes in either potential recipient strain that might give rise to meta- or

TABLE 5

CONCENTRATION SENSITIVITY TO PHENOLIC SUBSTRATES

Organism	P9	P9N	P1	P1N	M9	M9N	M1	M1N	LN	LA
JPT3	+	-	+	-	+	-	+	-	-	+
JPT3-4	+	-	+	-	+	-	+	-	-	+
JPT8N-80	+	+	+	+	w*	-	w*	-	+	+
J3-102-A	-	-	w*	-	-	-	w*	w*	+	+
J3-102-B	+*	w*	+	+*	+*	-	+	+*	+	+
J3-102-C	-	-	w*	-	-	-	w*	w*	+	+
J3-102-D	-	-	w*	w*	-	-	w*	w*	+	+
J3-102-E	-	-	w*	w*	-	-	w*	w*	+	+
J3-102-F	-	-	w*	w*	-	-	w*	w*	+	+
J3-102-G	NT	NT	NT	NT	NT	NT	w*	w*	+	+
J3-102-H*	NT	NT	NT	NT	NT	NT	w*	w*	+	+
J3-8R1-A	+	+	+	+	+*	+*	+	+	+	+
J3-8R1-B	+	+	+	+	+*	+*	+	+	+	+
J3-8R1-C	+	+	+	+	-	-	w*	w*	+	+
J3-8R1-D	+	+	+	+	-	-	w*	w*	+	+
J3-8R1-E	+	+	+	+	-	-	w*	w*	+	+
J3-8R1-F	+	+	+	+	-	-	w*	w*	+	+

P = Para-cresol
M = Meta-cresol
N = Neomycin
L = Luria

A = Agar
9 = 900ppm
1 = 100ppm
NT = Not Tested
- = No growth

w* = weak growth, thin and small colony
+ = strong growth, thick, large colony
+* = strong growth, small colony

ortho-cresol or phenol hydroxylases (HXs), or to any 2,3-oxygenase (2,3OX) activities. A few *PMOcre*$^{+}\emptyset^{+}$ exconjugants were obtained which still retained PAS102N's *thr*$^{-}$ phenotype, but were now just barely able to survive on cresol or phenol concentrations--up to 450 ppm--which had been lethal to PAS102N (Souder, unpublished observations). No significant oxygenase activities of any kind were detectable. A comparison of phenolics tolerances for all strains and putative recombinants is contained in Table 5.

In mate exconjugants, generally, there appeared to be at least four recognizable categories:

GROUP I: those whose hydroxylase (HX) and oxygenase (OX) activities resembled the donor's;

GROUP II: those whose HX and OX activities resembled the recipient's (no apparent transfer of degradative genes);

GROUP III: those exconjugants demonstrating no significant, inducible donor HX (for meta-, ortho-cresol or phenol), but having significant levels of 1,2OX, 2,3OX or 3,4OX activities characteristic of the donor;

GROUP IV: those with at least some donor-characteristic HX activity, and donor OX activities.

For the JPT3-4 x PAS102N cross, of eight exconjugants isolated, four (J3-102A, B, C, and D) were tested in the oxygen monitor. In succinate media, all four required threonine at 40 ppm for growth, establishing their identity as recombinant offspring with high certainty, and indicating

that any *cre* genes transferred were either not linked to the JPT3-4 chromosome, or came prior to the *thr* cistron on the donor chromosome.

Table 6 depicts the responses of three-day succinate/threonine cultures to the test substrates following a two-hour induction on a selected induction substrate. For JPT3-4 x PAS102N exconjugants, J3-102A and C expressed only a low level of hydroxylating activity for all three cresols and phenol, but operated very effectively on the catechols, placing them in Group III. J3-102B expressed a strong para-cresol induced HX for para-cresol, but only limited HX activity on the other cresols and phenol. When other phenolics served as inducers, the overall HX activity was moderately low, even for para-cresol. Yet, again, the catechol oxygenase (OX) activities were very strong, placing J3-102B in Group IV. J3-102D resembled parental recipient PAS102N in that virtually no inducible HX or OX activities were detectable, placing it in Group II. Concentration sensitivities of these exconjugants is depicted in Table 5.

Exconjugants obtained from JPT3-4 x JPT8N-80 mates were of at least two types: One, represented by J3-8R1E, grew well on para-cresol; gave oxygen monitor results compatible with possessing para-cresol HX; showed no meta-, ortho-cresol nor phenol HX activity to speak of, even when the strong inducer, ortho-cresol, was included in growth media; yet had strongly inducible OX activities for the

TABLE 6

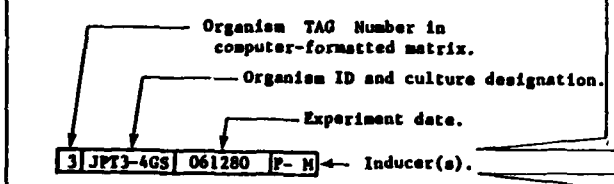
EXCONJUGANT INDUCTION DATA*

INDUCER: GROUP TWO CULTURE I.D.	FPM	PARA	META	ORTHO	PHENOL	BENZ	CAT	3MCAT	4MCAT	PROCAT
1- J3Δ102D-071180-P -TH	83	12.12	.00	.00	.00	.00	2.09	3.55	10.45	8.36
2- J3Δ102D-072180-ST-↑M	83	19.77	13.18	10.19	7.39	1.80	38.55	20.77	64.91	1.00
3- J3Δ102D-072580- S-↑F	83	8.44	2.09	4.72I	.00	22.86I	2.65	.72	13.03	6.25I
1-J3Δ8102F-061280- P-	165	17.20	12.42	16.67I	.96	13.33I	.96	.00	19.11	118.49
2-J3Δ8RI-F-061280- P- O	165	172.18	35.51	.00	6.25I	1.08	.00	3.23	103.31	91.47
4-J3Δ8RIF -062480- P- F	206	62.47	31.93	3.33	7.14I	.00	2.70	9.72	26.30	105.20

INDUCER: GROUP THREE CULTURE I.D.	FPM	PARA	META	ORTHO	PHENOL	BENZ	CAT	3MCAT	4MCAT	PROCAT
1- J3Δ8RIE-061280- P-	206	111.20	56.04	14.01	6.65	30.65	83.63	104.37	274.94	108.40
4- J3Δ8RIE-070280- P- M	83	10.51	9.20	1.31	.00	1.31	144.57	81.49	546.74	63.09
5- J3Δ8RIE-070180- P- O	83	29.54	12.31	4.92	4.92	.00	123.07	120.61	390.76	54.15
1-J3Δ102A -071180-P -TH	83	76.28	15.26	10.90	16.13	2.18	74.10	65.38	239.74	60.00
2- J3Δ102A-071880-M -ST	83	30.00	4.32	3.45	6.39	2.42	98.20	51.65	177.91	3.45
4- J3Δ102A-072680-ST-↑F	83	71.39	30.43	37.45	64.37	9.36	283.24	225.89	812.26	2.34
1- J3Δ102C-071180-P -TH	83	26.35	100.00I	.00	2.31	.00	38.37	43.92	167.35	92.46
2- J3Δ102C-071880-M -ST	83	18.00	2.00	.00	.80	.60	13.80	9.00	132.00	1.80
4- J3Δ102C-072680-ST-↑F	83	92.81	45.39	40.83	57.06	5.58	154.69	111.07	403.21	2.54

INDUCER: GROUP FOUR CULTURE I.D.	FPM	PARA	META	ORTHO	PHENOL	BENZ	CAT	3MCAT	4MCAT	PROCAT
1- J3Δ102D-071280-P -TH	83	170.13	23.47	29.33	20.53	5.87	149.60	76.27	305.07	99.73
2- J3Δ102D-071780-MS-TH	83	69.87	31.90	36.46	59.24	3.04	118.48	113.92	232.41	1.52
4- J3Δ102D-072580-ST-↑F	83	19.77	11.45	12.49	16.65	.00	41.63	38.51	169.44	4.16

CULTURE I.D. KEY



INDUCER ABBREVIATIONS

P = para-cresol B = benzoate
M = meta-cresol A = acetate
O = ortho-cresol S = succinate
F = phenol TH= threonine

↑ preceeding an inducer means the organism was raised on an alternate carbon source, but induced 2 hrs prior to harvest on the phenolic following ↑.

*INDUCTION DATA given in microliters of O_2 utilized per hour per mg of cells dry weight; all values are *minus endogenous respiration O_2 utilization.*

(*I*) following an entry indicates substrate inhibited endogenous oxygen consumption by the % indicated.

catechols and PROCAT. These results placed J3-8R1E in Group III. A second exconjugant, J3-8R1F, which had grown out only poorly on the meta-cresol/Neomycin selection plate, showed no significant differences between its induction patterns and those of the recipient parent, JPT8N-80, and therefore could be placed in Group II. Two other isolates, J3-8R1A and B, grew well on all three isomers of cresol and on phenol, but were not tested in the oxygen monitor. However, since M900-growing colonies on a plate turned yellow when sprayed with 4MCAT, and since their growth performance on cresol agar media was indistinguishable from donor-parent JPT3-4, the two have been allocated to Group I. Two remaining exconjugants, which gave selective media growth responses identical to those of J3-8R1E and F (*i.e.*, small but definite colonies on M100-Neo), were isolated but not further examined. All six isolates, however, were "fingerprinted" for their plasmid DNA patterns, as discussed below, and have been freeze-banked for future study.

Enzyme extraction procedure development results. Hegeman (1966) reported variances between successive spectrophotometric assays on "identical" extracts to be $\pm$ 30%, with the principal sources of error being physical ones; *i.e.*, inaccuracies in protein determination results, degrees of enzyme inactivation under storage or handling conditions,

etc. These data show a similar variability, but nonetheless indicate the existence of activities and of trends, even when the data is viewed strictly qualitatively, as it is reported in Table 3. The main stumbling block to quantifying enzyme activity results was the unreliable quantification of protein concentrations in extracts in the procedure developed below.

During the tracing protocols used to discover the fate of 2,3OX activities in the Hatcher technique, several characteristics of JPT3-4's oxygenases were determined (see Figures 3 & 4):

1. All 2,3OX activities, which should have yielded absorption peaks for ring-fission products of the catechols in the 370-390 nm range, were sensitive to oxygen;

2. Principal 2,3OX activities were located in the pellet fraction of sonicated JPT3-4 cell preps;

3. A weak 2,3OX activity, relatively specific for 4MCAT, sensitive to oxygen (90 sec. lifetime when sonicated supernatant was exposed to air at 30°C.), was localized in the soluble portion of the cell sonicate;

4. A strong 2,3OX activity working on all three catechols, and relatively insensitive to oxygen (15 min. lifetime when exposed to air at 30°C.), was localized in the resuspended sonicate pellet;

5. All 2,3OX activities were destroyed by a heat pulse step (50°C. for three minutes);

6. All 2,3OX activities were destroyed by precipitation with protamine sulphate;

7. 2,3OX activities were not significantly restored by treatment with $NaBH_4$, as recommended by Bayly and Wigmore (1973);

8. Acetone added to "reactivated" preps, or use of a phosphate buffer 1 mM in dithiothreitol, did not improve lifetimes of 2,3OX activities in either extract fraction;

9. Treatment of washed cells with 20% SDS totally lysed the cells but destroyed all detectable enzyme activities.

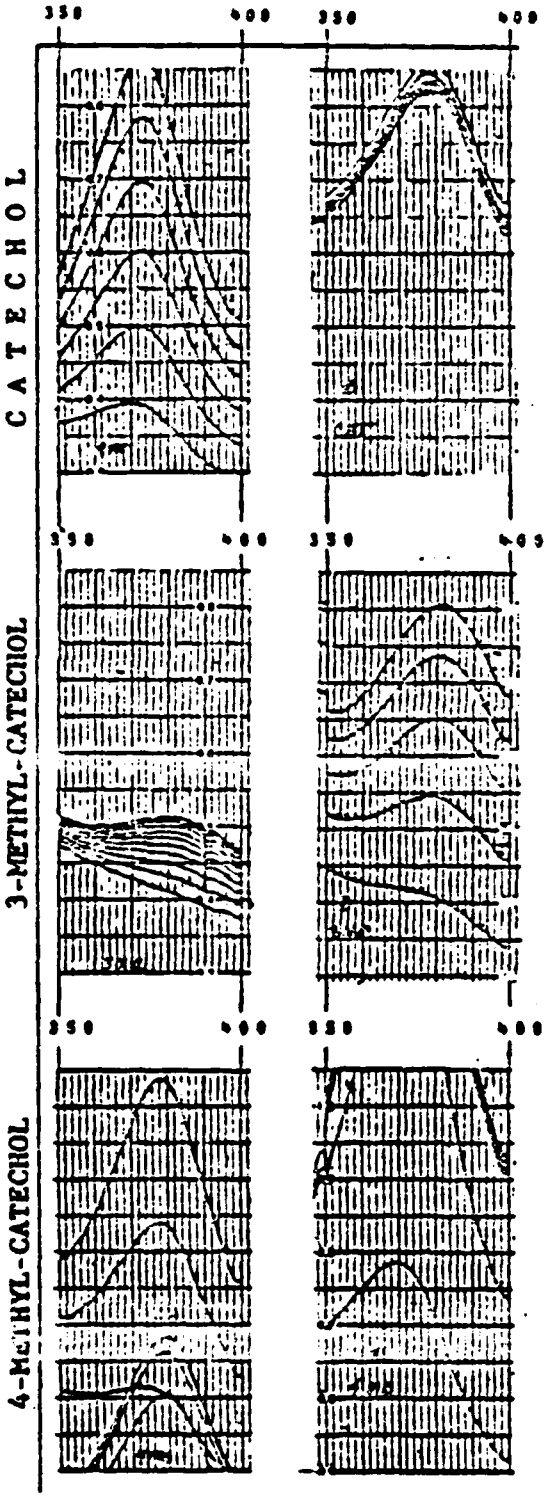
Failure to isolate the oxygenase activities from the subsequent step in "meta" ring-fission can be seen in Figure 16 . Bayly and Wigmore (1966) introduced the tactic of adding ten times the normal test concentration of 3MCAT to their spectrophotometric test cuvettes in order to correct for the rapid breakdown of 2-hydroxy-6-keto-2,4-heptadienoate, the product of 2,3-fissioned 3MCAT. Hatcher (1977) did not use this procedure, but reported specific enzyme activities based on only the first 60. seconds of reaction time for all catechol substrates, stating that rates were most reproducible within that early time frame. In this study, there was such a wide variation in the hydrolase activity (HMSH, Figure 1) following fission of 3MCAT that no attempt was made to calculate specific activities. Instead, Table 3 contains the columns labelled *1,2/3,4OX* and *2,3OX*, indicating whether or not activities characteristic of these oxygenases were detected. Figures 15 through 17 are included to illustrate the typical spectrophotometric absorbance curves given by the enzyme preparations.

Not all questions of pathways at work could be resolved by looking at spectrophotometric results. Although the expected peaks were known for most of the metabolites

JPT3-4 activity on PROCAT was never visualized. However, using identical procedures, the action of an oxygenase was easily observed on PROCAT with JPT8N-80 extracts.

FIGURE 15

JPT3-4 CATECHOL "META" FISSION PRODUCTS



Spectrophotometric scans in the visible, from 400 down to 350 nm, clearly show the characteristic absorbance patterns of "meta" (2,3) fission products of the catechols.

Extract used in lefthand column came from JPT3-4 induced on META-CRESOL; on the right, from JPT3-4 induced on PHENOL.

Only the pellet fraction of the sonicate, obtained under anaerobic conditions, was used in the tests.

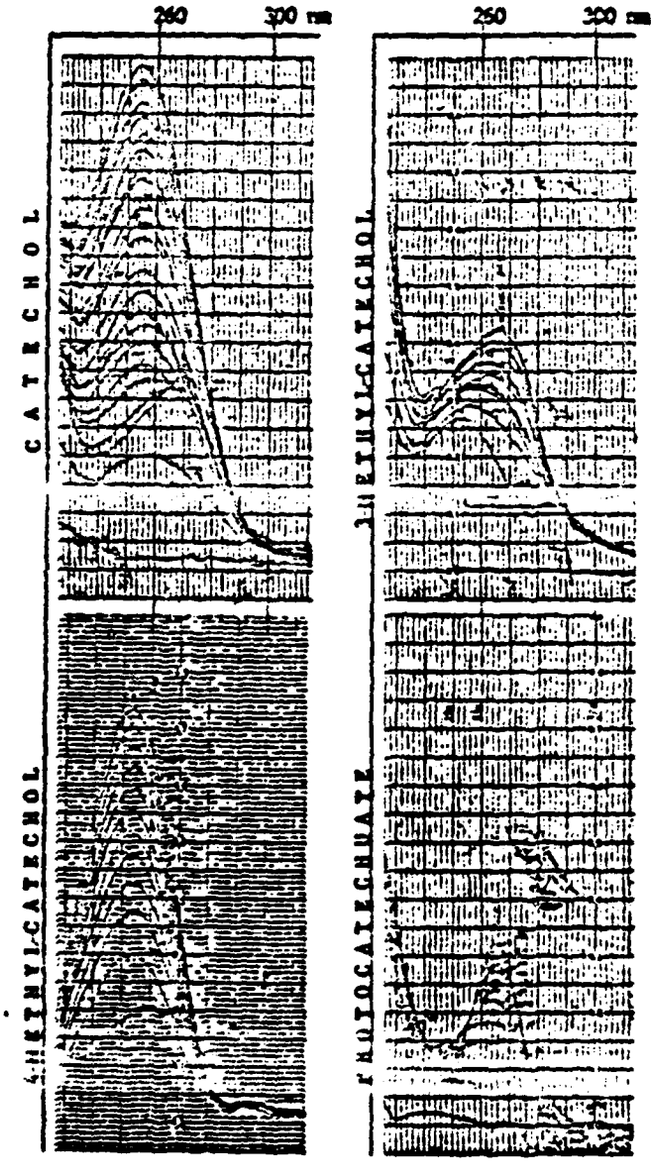
Assays were conducted at 25°C., in .05M phosphate buffer at pH 7.5. Enzyme was stored until used in a sealed phial under nitrogen at 0°C. Reaction cuvettes contained 3 ml phosphate buffer, .1umole of the test substrate except for 3MCAT, for which 1 umole was added. 0.05 ml of extract was added to initiated the reaction. Scans were made at 53 sec. intervals.

Under these conditions, no UV activity was observed in any of the JPT3-4 extracts, with the exceptions illustrated in Figure 18.

Decreasing spacing between sequential scans, evident in several sets, may indicate a slowing of reaction rate due to either exhaustion of reaction components, or to increasing removal of the observed product, or to a combination of both.

FIGURE 16

UV ACTIVITY IN JPT3N-80 EXTRACTS



UV ABSORBANCE SPECTRA for JPT3N-80 nitrogen-protected enzyme extracts acting on the catechols and protocatechuate in phosphate buffer, pH 7.5, with EDTA (see text).

JPT3N-80 cells were raised and induced on PARA-CRESOL.

UV activity was found in both pellet and supernatant.

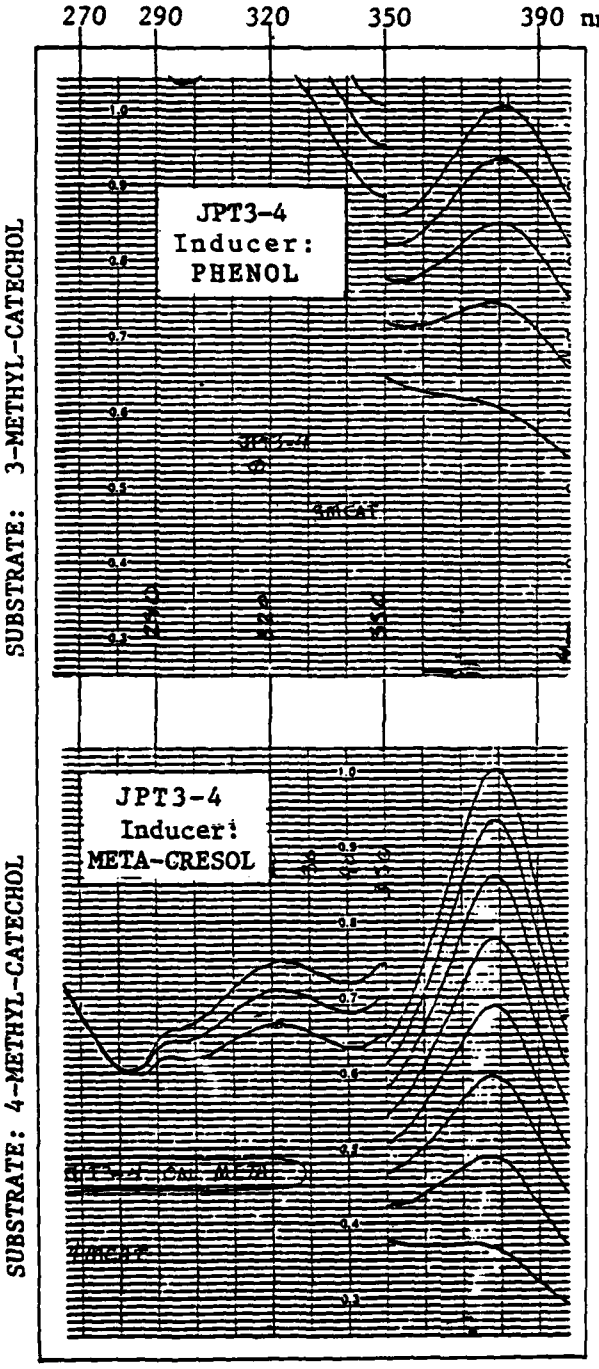
Peaks seem to drift toward a final value away from the 260 nm peak characteristic of the extract alone.

PROTOCATECHUATE's complex absorbance pattern appears due to the disappearance of the substrate—which absorbs at 247 and 285 nm, as shown in Figure —and the appearance of a product at an indeterminate intermediate wavelength.

Absorbance patterns are similar to those reported by Dagley and Patel (1957) and by Hayly et al. (1966) for the maxima of "ortho" fission products for the catechols. No visible wavelength activity was observed in JPT3N-80 extracts under any induction conditions.

FIGURE 17

INTERMITTENT DETECTION OF UV ACTIVITY IN JPT3-4's WITH STRONGLY INDUCED "META" PATHWAYS



In some extracts of JPT3-4 showing high 2,3-oxygenase activities, appreciable amounts of activity in the UV could also be detected near 290 and 320 nm. These peaks were most noticeable for those cultures induced on PHENOL and on META-CRESOL, when extract was injected into cuvettes containing 3- and 4-methyl-catechol in phosphate buffer at pH 7.5 (Hatcher, 1977).

The activity could represent a buildup of products further down the "meta-fission" pathway, although no spectroscopic absorbance peaks were listed for those compounds (Figure 2) under conditions similar to those used here. Hopper and Taylor (1975) describe a gentisate pathway potentially able to act on methyl-catechols, yielding fission product peaks near 320 nm. Further investigation of this phenomenon was beyond the scope of this study.

Plasmid DNA Studies

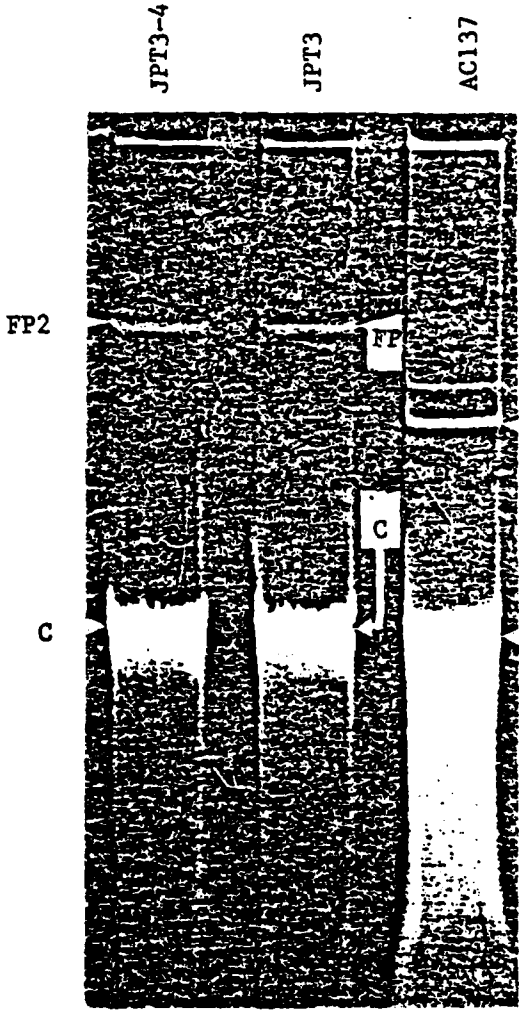
All of the plasmid DNA preparation methods tried were able to provide a consistent picture of the plasmid populations of the test organisms. JPT3-4's large plasmid, which migrated well behind the 70 Mdal TOL plasmid in Chakrabarty's AC137 (see Figure 18), was first detected in phenol extraction preps (Guerry *et al.*, 1973; Meyers *et al.*, 1976). A subsequent switch to a less objectionable solvent system--the 24:1 chloroform:isoamyl alcohol system suggested by M.P. Moyer-- gave no detectable differences in recovery efficiencies. However, the Hansen and Olsen (1978) alkaline denaturation/PEG precipitation method was found to yield less contaminating chromosomal DNA fragments; and the yield appeared to be better than yields from the other preps, based on the densities of plasmid bands in agarose gel electrophoresis preparations.

Occasionally, low molecular weight bands can be resolved in the "front-running" halo of nucleic acid fragments near the base of two-to-three hour gels (see Figure 19). Whether these bands are small plasmids or RNA fragments has not yet been resolved.

Donor, recipient, and exconjugant plasmid populations. Figures 18 & 19depict the agarose gel plasmid bands associated with the donor, JPT3-4; the recipients, JPT8N-80 and PAS102N; and

FIGURE 18

JPT3-4 FP2 BAND COMPARED TO TOL



RUN CONDITIONS:

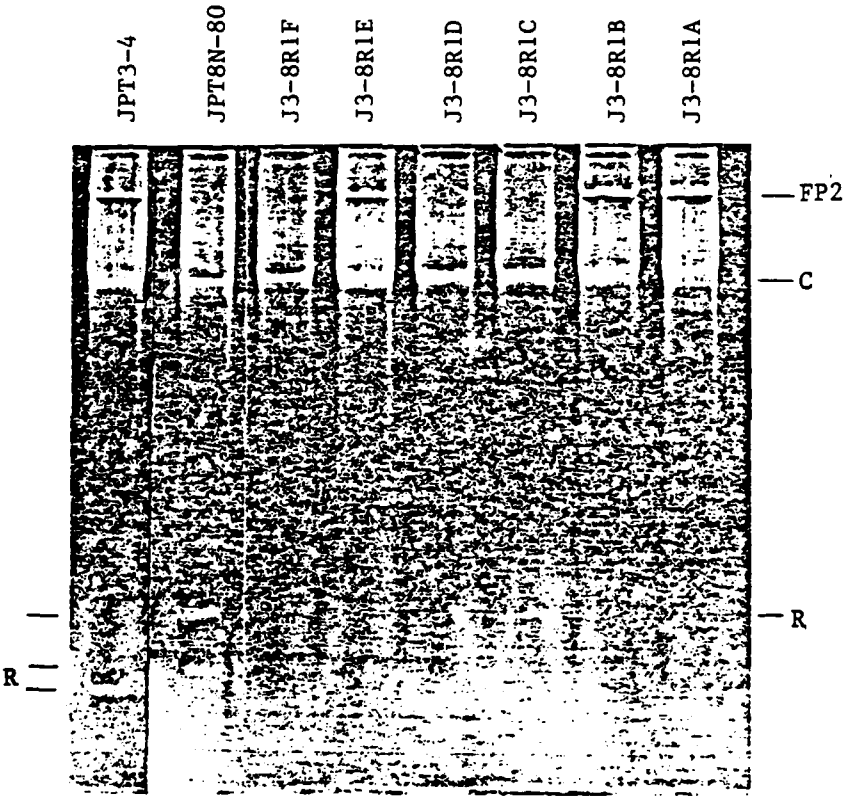
3mm 0.7% agarose gels in E-buffer, 2 gels

2.5 hr 120V 73 mA

FP2 = sex factor plasmid from PTO13; TOL = degradative plasmid coding "meta" fission pathway for toluene (obtained from A.M. Chakrabarty); C = linear chromosomal DNA fragments.

FIGURE 19

VISUALIZATION OF MATE EXCONJUGANT PLASMID DNA



RUN CONDITIONS: 3mm 0.7% agarose gels
E-buffer 2 hr 100V 30 mA

FP2 = sex factor plasmid, transferred from JPT3-4 into exconjugants isolated on M100 Neo plates at T+105 min.; C = linear chromosomal DNA fragments; R = probable RNA bands, not always seen, probably dependent upon individual prep handling.

the exconjugants, J3-8R1A through F, and J3-102A through H. In virtually every DNA prep there is a somewhat diffuse leading band, which has been shown to co-migrate with purified lambda-phage DNA (Olive, unpublished results). This band has been cut with EcoRI endonuclease, yielding in excess of twenty distinct fragment sizes, a pattern totally unlike that of lambda. It is not yet clear exactly what this band represents, nor is it known if the bands in the different organisms here contain similar fragments.

The electrophoresis pattern for JPT3-4 is shown in Figure 18. No accurate measurement has been made of the large plasmid trailing the lambda-weight diffuse band. However, when electrophoresed with a prep from *Ps. putida* AC137 (data not shown), the JPT3-4 plasmid migrated more slowly than the TOL plasmid carried by AC137 (Williams and Murray, 1974; Williams and Worsey, 1976; see Figure 18). Williams has estimated TOL at 70 Mdal. The JPT3-4 plasmid was introduced into the J-series during a mating of *Ps. aeruginosa* J1 with *Ps. aeruginosa* PTO13. PTO13 carried the FP2 sex factor plasmid. Electrophoresis of the parental and exconjugant strains showed that JPT3-4's plasmid band migrated at very nearly the same rate as PTO13's FP2 (data not shown).

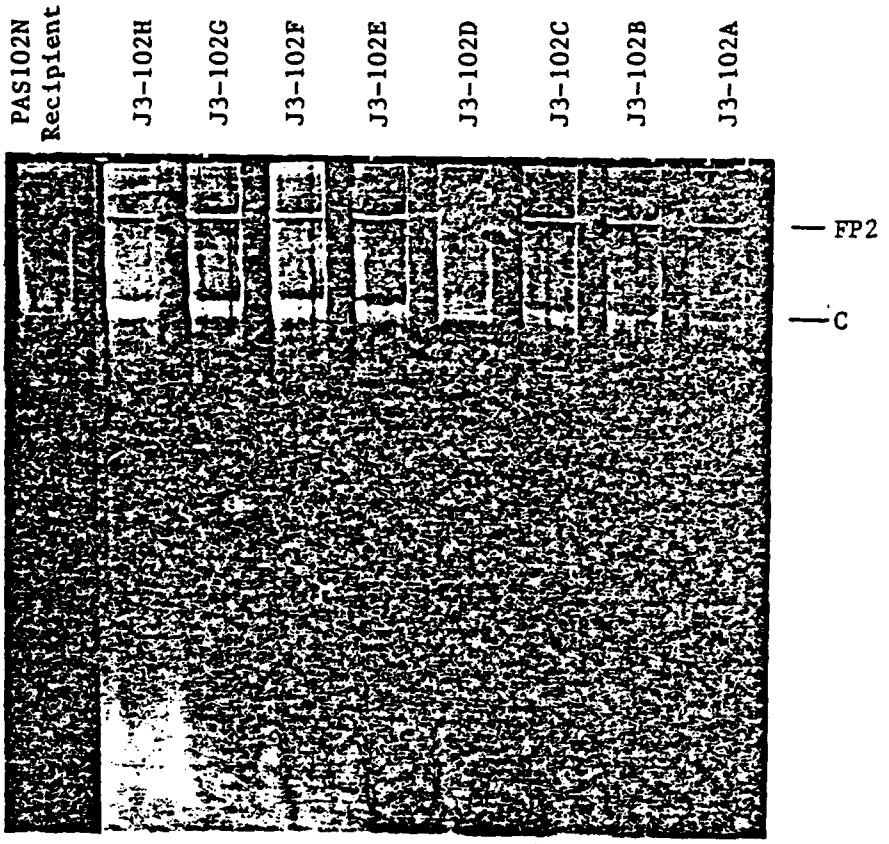
JPT8N-80, except for the diffuse front band, did not yield any sign of either large or small plasmids in any of the various preps administered. PAS102N DNA extractions were

also devoid of plasmid bands. The agarose gels of the two series of exconjugants, however, clearly demonstrated the transmissible nature of the JPT3-4 plasmid (Figures 19 and 20). The relationship between the presence of the JPT3-4 plasmid in an exconjugant, and the presence of inducible and/or constitutive cresol, phenol, benzoate or protocatechuate oxidative enzymes is illustrated in Table 3 .

Exconjugants were isolated both with and without the JPT3-4 plasmid band (plasmid pJP1), on a selective media composed of 100 ppm meta-cresol, 40 ppm threonine, and 400 ppm Neomycin sulphate in 2% BMS agar. Colony size, as a measure of phenotypic expression of transferred genes, appears in Table 5 also. For JPT3-4 x JPT8N-80 exconjugants, only J3-8R1A and B appeared to fully express the cresol-degradation capability, growing well on all of the phenolic test media. J3-8R1C through F all appeared as marginally-surviving colonies on the selective media, although each was able to grow well on the para-cresol isomer and on benzoate. As mentioned in the section on enzyme studies, the exconjugants broke into four groups with respect to possession of para-cresol HX activity, meta-ortho-phenol HX activity, and 1,2OX *versus* both 1,2OX and 2,3OX activities. All exconjugants bearing pJP1 possessed the 2,3OX activities acting on all three catechol substrates. Some also possessed HX activities for para-cresol and the meta-ortho-cresol-phenol

FIGURE 20

VISUALIZATION OF MATE EXCONJUGANT PLASMID DNA



RUN CONDITIONS: 3mm 0.7% agarose gels
E-buffer 2.5 hr 100V 30 mA

FP2 = sex factor plasmid, transferred from JPT3-4 into exconjugants isolated on M100 thr 20 Neo media at T+60 min.; C = linear chromosomal fragments.

group, while others had only the para-cresol HX activity. Those exconjugants without the plasmid band exhibited no 2,3OX activities and no meta-ortho-cresol-phenol HX activity, appearing identical to their recipient parent, JPT8N-80. JPT3-4 x PAS102N exconjugants exhibited the same relationship between the presence of pJP1 and the possession of 2,3OX activity and meta-ortho-cresol-phenol HX activity (Table 3).

DISCUSSION

Pseudomonas aeruginosa J1 had been studied in this laboratory by Hatcher (1977) because it had shown superior ability to utilize all three isomers of cresol in tests leading to development of a laboratory-scale pilot plant for degrading phenolic wastes (Cobb *et al.*, 1979). Hatcher identified a "meta-pathway" in J1 using both whole-cell oxygen consumption and enzyme extract spectrophotometric data, but found no evidence of 1,2-oxygenase or "ortho-pathway" activity. He did report, however, a peculiarity of para-cresol induced cultures: When induced on that isomer, abnormally low levels of whole cell activity on meta- and ortho-cresol were evident, together with low activities on the related catechols (CAT and 3MCAT). Yet, when a 2°C., low ionic strength phosphate buffer wash was used in place of the normal 24°C. BMS buffer wash during harvest, some activity on all four of these substrates was detected spectrophotometrically and in the oxygen monitor. Hatcher explained these findings in terms of inducible permease (symport and antiport) systems acting differentially on the cresol isomers (para- *versus* meta- and ortho-cresol) and on the catechol derivatives (4MCAT *versus* 3MCAT and CAT), which were adversely affected by the combination of osmotic shock and cold. He concluded that para-cresol did induce HX and 2,3OX activities of low specificity, which could act

on all three isomers and all three catechols, but that a permease exclusion (antiport) system prevented entry of the meta and ortho isomers, as well as 3MCAT and CAT. When these antiport systems were damaged, passive transport brought the materials into the cells, where the induced enzyme systems could then act upon them. This point is brought up here because it has some bearing on establishing the degree of genetic similarity between J1 and its $FP2^{+}$ derivative strain, JPT3-4.

Examination of a page of data taken from Hatcher's Master's Thesis (Table 7), with an added set of columns for JPT3-4, shows how widely the numbers differ for these organisms under similar experimental conditions. The major difference in treatment was the use of cold BMS for both washes, and 30°C. BMS in the oxygen monitor for the JPT3-4 runs. When JPT3-4 cultures were harvested in room temperature BMS and immediately tested on the oxygen monitor, para-cresol, 4MCAT and PROCAT activity remained high, but all other hydroxylase and catechol activities decreased markedly. If such a room temperature-washed culture was then chilled briefly in cold BMS, then rewarmed and examined in the oxygen monitor, no change in this picture occurred (Egan, unpublished data). It seems possible that an alternative explanation for the para-cresol "cold/osmotic shock" peculiarities might be offerred: 2,3OX activity in JPT3-4 was markedly oxygen-sensitive and heat-labile. It might

TABLE 7

COMPARISON OF J1 AND JPT3-4 WHOLE CELL O_2 DATA

CELLS WASHED IN	CELLS TESTED IN	TEST SUBSTRATE	INDUCER		
			PARA	META	ORTHO
24°C, BMS ¶	30°C, BMS	Para-cresol	285.1 *	68.5 *	70.1 *
		Meta-cresol	0	59.7	59.7
		Ortho-cresol	0	60.6	59.7
2°C, PB ¶	30°C, PB	Para-cresol	57.9	23.4	40.7
		Meta-cresol	26.0	14.7	32.0
		Ortho-cresol	26.0	14.7	34.6
0°C, BMS ✱	30°C, BMS	Para-cresol	278.1	113.2	348.8
		Meta-cresol	86.6	67.1	186.1
		Ortho-cresol	56.8	67.1	181.0
24°C, BMS ¶	30°C, BMS	Catechol	0	84.8	83.0
		3-M-Catechol	0	86.5	81.3
		4-M-Catechol	83.9	97.8	128.1
2°C, PB ¶	30°C, PB	Catechol	19.9	225.0	346.1
		3-M-Catechol	17.3	225.0	326.2
		4-M-Catechol	32.0	392.0	426.6
0°C, BMS ✱	30°C, BMS	Catechol	90.5	377.3	1072.9
		3-M-Catechol	63.4	276.7	642.0
		4-M-Catechol	284.6	578.6	1094.8

¶ These results for J1 extracted from Hatcher (1977).

✱ These results for JPT3-4 extracted from Table 4.

BMS is the mineral salts buffer of Olive (1975).
PB is the phosphate buffer of Hatcher (1977).

* RESULTS GIVEN IN MICROLITERS OF OXYGEN CONSUMED PER HOUR PER MG CELLS, DRY WEIGHT.

therefore be expected to show sensitivity to storage conditions even within the undamaged cell. If para-cresol in fact induced smaller amounts of the enzymes specific for hydroxylating para-, meta-, and ortho-cresol *to catechols*, and also smaller amounts of 2,3OX activities (and perhaps the subsequent "meta" pathway enzymes as well, as Hatcher's data seems to show), then further erosion of activity during a warm buffer wash and storage condition prior to testing may have resulted in the very low levels observed by Hatcher in para-induced cells. The "cold/osmotic shock" condition with chilled phosphate buffer might have protected the low-level activities, allowing them to be detected in the oxygen monitor. In JPT3-4, this set of explanations fits the data very well. A cold BMS wash resulted in a picture looking very much like a composite of Hatcher's results--with both high para-cresol hydroxylation and 4MCAT oxidation, and moderate meta- and ortho-cresol hydroxylation accompanying moderate CAT and 3MCAT oxidation.

Spectrophotometrically, Hatcher observed no "ortho" fission peaks for any of his catechol substrates. When the Hatcher technique (modified after Bayly and Wigmore, 1973) was used with JPT3-4, no spectrophotometric activity at all was observed. In this respect, the donor organism in this study differed strongly from the recorded responses of *Pseudomonas aeruginosa* J1. After a technique rescuing ortho fission activity was worked out, the results of

catechol oxidations could easily be detected in para-induced cell extracts. However, the peaks observed were never as strong as those generated during the oxidation of catechols by meta-, ortho-, or phenol-induced extracts. But, as Hatcher had reported, no "ortho" ring-fission peaks were detectable for any substrate, including protocatechuate.

This last fact gave a hint of what might have been happening. JPT8N-80 cell extracts, using the same 0.05M $KH_2PO_4 \cdot Na_2HPO_4$ buffer system at pH 7.5 (Hopper and Taylor, 1975), readily demonstrated UV absorption peaks for all three catechols and for PROCAT, typical of 1,2- and 3,40X fissioning of the ring. Para-cresol induced JPT3-4 showed an equal ability to oxidize PROCAT in the oxygen electrode. Yet the sonicated extracts were consistently void of PROCAT activity. If PROCAT activity was invisible to the procedure, then it was possible that activities other than the low observable 2,30X acting on the catechols might also be escaping detection.

Other pathways for para-cresol were known to exist. In 1952 Smith *et al.* described a pseudomonad apparently metabolizing para-cresol via parahydroxybenzyl alcohol, parahydroxybenzaldehyde, and parahydroxybenzoic acid. MacDonald *et al.*, (1954) isolated a protocatechuic oxidase converting PROCAT to *cis-cis*-β-carboxymuconic acid from *Ps. fluorescens* extracts. Stanier and Ingraham (1954) showed that this PROCAT oxidase did not seem to have cofactor

requirements. Dagley and Patel (1957), using an organism identified as related to *Ps. cruciviae*, isolated a PROCAT oxidase as part of a chain of events leading from para-cresol to an unknown compound (later identified as β-keto-adipic acid). This PROCAT activity required Fe^{2+} as a co-factor, and was relatively labile. Ornston (1966 a and b) described a protocatechuate oxygenase from a *Ps. putida* which was highly specific for PROCAT, with activity for catechol at about 2% of its target substrate rate.

An investigation by Keat and Hopper (1978b), built on several years of studies of para-cresol metabolism in *Ps. putida* N.C.I.B. 9869 (Hopper and Taylor, 1975; Hopper and Taylor, 1977; Keat and Hopper, 1978a), isolated two para-cresol-specific methyl hydroxylases, required for the first step in carrying para-cresol through the protocate-chuate 3,40X "ortho" fission pathway. These methylhydrox-ylases would not attack meta- nor ortho-cresol to any great extent. As is shown in Figure 14, a brief check of para-cresol and PROCAT induced JPT3-4 cells did reveal the induction of oxidizing activities for the intermediates in the para-cresol-to-PROCAT pathway, and for PROCAT, in a manner consistent with Stanier's (1947) principle of "simultaneous adaptation." It is also noteworthy that Dagley and Patel (1957) showed their organism's methyl-hydroxylase would attack 4MCAT's methyl group, and convert the compound to PROCAT before fissioning the nucleus; this

PROCAT-fissioning activity would not act on catechol, nor on 3MCAT. Furthermore, the ring-hydroxylating activity present in para-induced cells was specific for parahydroxybenzoic acid, and was not involved in hydroxylation of para-cresol directly.

Based on these reports, and on the results of the tests delineated in Figure 14, it is highly probable that para-cresol induced a pathway in JPT3-4 that carried para-cresol through parahydroxybenzylalcohol, parahydroxybenzaldehyde, parahydroxybenzoic acid (via methylhydroxylations), then, via ring-hydroxylation, to protocatechuate, and down the "ortho" fission pathway (Figure 5). It also seems likely, from 4MCAT oxidation figures in acetate-grown cells, and in para-cresol induced cultures, that an activity specific for 4MCAT can exist in JPT3-4, which does not result in its immediate fissioning (as by a 2,30X or 1,20X). If, as in the Dagley and Patel organism (1957), a methylhydroxylase acts on 4MCAT to carry it to PROCAT, oxygen utilization would be detectable, even in cases where no ring-fission took place. The fact that 2,30X activity for 4MCAT, CAT, and 3MCAT was detectable in enzyme extract reactions with para-cresol induced cells would seem to show that the "meta" fission pathway was also induced, albeit to a low extent, by para-cresol. Thus, the continued favoring of 4MCAT as a substrate by JPT3-4, even when a fully-induced "meta" fission pathway was clearly operating, might

have been due to a continuing methylhydroxylation activity also operating on 4MCAT.

The existence of such methylhydroxylases specific for para-cresol also implies that, if any type of antiport or symport systems are involved in controlling the movement of the cresols or catechols across JPT3-4's membrane, there might indeed be some kind of para-cresol specific symport mechanism, as Hatcher had suggested. The varying ratios of hydroxylase and oxygenase activities in all of the test organisms, particularly when multiple inducing substrates were used in culture growth, underscores the possibility that it was not merely hydroxylase or oxygenase activities being monitored with whole cells, but also any facilitative or inhibitory transport mechanisms, modulated by alterations in membrane components whose existences would be invisible to experimental protocol.

A full alternate explanation for both J1's and JPT3-4's para-cresol metabolic peculiarities, then, would seem to be that para-cresol induces a specific symport system for para-cresol; a system of methylhydroxylases acting both on para-cresol and 4MCAT--carrying both to PROCAT and through a PROCAT-specific 3,4-oxygenase--but inducing a low level of 2,3-oxygenase and subsequent "meta" pathway activity, and an equally low level of cresol ring hydroxylase activities. From cultures grown on acetate and on succinate, it also appears that the para-cresol/4MCAT methylhydroxylase activi-

ties are constitutively induced at moderate levels, although all oxygenases show virtually no constitutive activities.

These data and explanations would seem to support the contention that JPT3-4, in spite of what initially appeared to be striking differences in metabolic characteristics compared to *Pseudomonas* J1, is in fact very similar to its parent strain. Acquisition of the FP2 plasmid from PTO13 may or may not have added the PROCAT-associated systems, but the interpretation of this experimenter is that such systems were present in J1 originally, but not revealed by the experimental protocol.

JPT8N-80 demonstrated ring-fission activities on CAT and 4MCAT when induced on benzoate alone, on para-cresol with phenol, or on benzoate mixed with other phenolics or acetate. Even 3MCAT was slowly metabolized by cell extracts, all giving spectrophotometric absorbance maxima at 254 nm. This figure is significantly different from the expected 1,2-oxygenase peaks of 260 nm reported by Hegeman (1966) and Feist and Hegeman (1969) for *cis,cis*-muconate's λ_{max}. JPT8N cultures induced on para-cresol alone; or para-cresol with acetate, meta-cresol, or with ortho-cresol, gave no UV signatures for any catechol, but did yield strong curves for PROCAT. These latter inducers were also the combinations showing strong 4MCAT and PROCAT oxidizing activities on the oxygen electrode tests (Table 4), but very little oxidation of CAT or 3MCAT. It is likely that JPT8N-80's "ortho" path-

way for 1,2-fission of catechols is induced only by catechol, which may be created in cells grown on benzoate by an induced benzoate oxidase; or to a limited extent by growth on phenol when para-cresol is also present. The para-cresol would primarily induce a methylhydroxylase system leading to para-hydroxybenzoic acid, followed by ring hydroxylation of this compound to protocatechuate (PROCAT), in a manner analogous to JPT3-4's activities. However, if phenol also diffuses into the cell, a low capability of the parahydroxybenzoic acid ring hydroxylase to hydroxylate phenol would give rise to catechol, and this in turn might serve to begin the stepwise "sequential induction" process characteristic of the "ortho" fission pathway, beginning with catechol 1,2-oxygenase (Ornston, 1966c; 1971). There is no evidence for a 2,3OX activity in JPT8N-80 at all. The curves for PROCAT oxidation in the spectrophotometer show an increasing peak at 265 nm, and two decreasing peaks, one at approximately 248 nm, the other around 290 nm. Interestingly, the absorbance peaks for authentic parahydroxybenzoic acid (247 nm) and parahydroxybenzaldehyde (285 nm) fall very close to those observed in the extracts. What they were doing there is another question. Scans made of "authentic" sodium protocatechuate used in all these experiments (Baker Chemical Co.) showed a bimodal peak with the greater maximum at 247 nm and the smaller peak at 285. It is very probable that the commercial prep was heavily contaminated with its two bio-

ogical precursors, which would explain the two disappearing peaks, and appearing central peak, in extracts metabolizing the commercial PROCAT preparation. The 265 nm increasing peak is therefore not necessarily significant, except as a portion of the spectrum not being affected by disappearance of products absorbing at the neighboring wavelengths (see Figure 21).

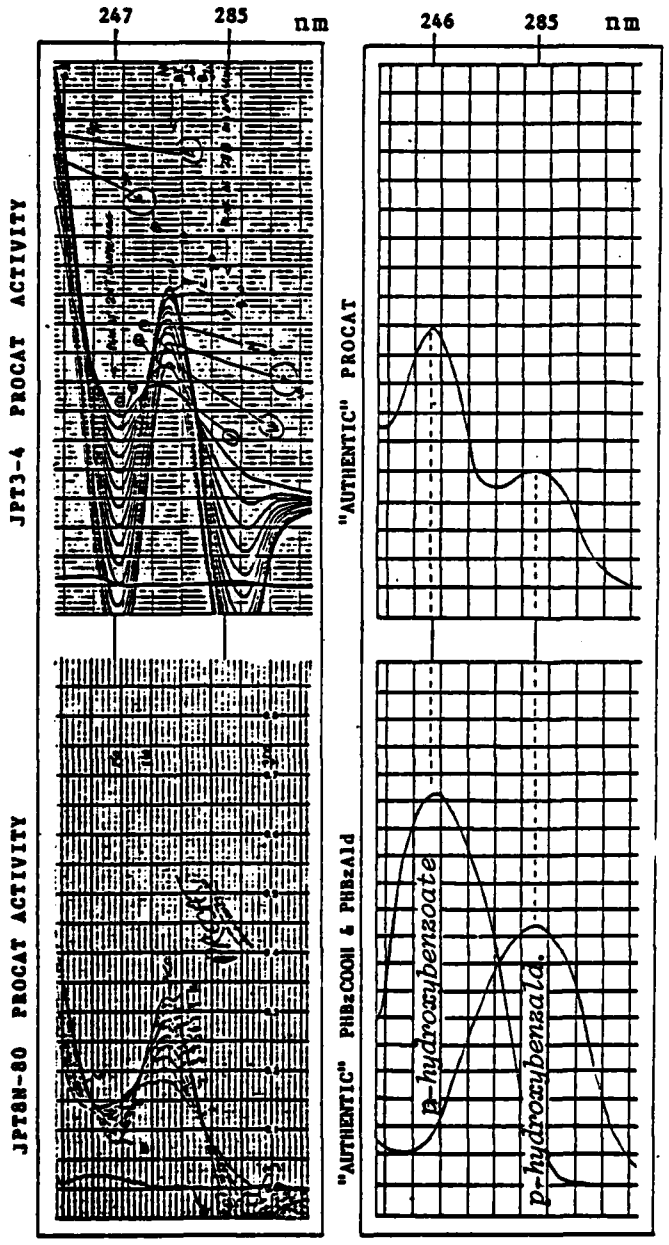
Genetic implications of mate exconjugant studies. Preliminary genetic studies of J1 had indicated that the ability to degrade the meta- and ortho-isomers of cresol could be lost from a population of this organism raised on rich carbon sources (Cobb *et al.*, 1977), and from cultures subjected to severe freeze-thaw cycles during storage and retrieval (Egan, unpublished observations). At the time, numerous biodegradative pathways for aromatic hydrocarbons were known, and some eight plasmids carrying genes involved in those pathways had been identified. Of particular interest were the P8 incompatibility group plasmids, TOL (Williams and Murray, 1974) and XYL (Friello *et al.*, 1976), which both carried genes specifying a "meta" ring-fission pathway for toluene and xylene, respectively. No one had suggested a plasmid involving the cresols, until in 1978, Dunn and coworkers (Hewetson *et al.*, 1978) published a report of the involvement of a plasmid, which they named pND50, in *Pa. putida* N.C.I.B. 9866, coding for the degradation of para-cresol via methylhydroxylases to protocatechuate, and

then down a 3,4OX "ortho" pathway. This plasmid was found to be transmissible, but could not be visualized by current means.

In contrast, strain J1 proved to be a female (F^-) organism (Cavalli *et al.*, 1953), and one apparently possessing restriction enzymes, since even mates with F^+ strains yielded detectable recombinants only after very long co-incubations (Rolfe and Holloway, 1966, 1968; Egan, unpublished observations). Any plasmid, if one was indeed involved in cresol metabolism in this organism, was apparently of the nontransmissible type (Holloway *et al.*, 1971; Chakrabarty, 1972; Clark and Warren, 1979). Since the *Pseudomonas* sex factor FP2 was known to mobilize both chromosomal and non-self-transmissible plasmid genes when transferred into an F^- strain (Holloway, 1955, 1956; Stanisich and Holloway, 1972), the $FP2^+$ strain PTO13 (derived in Holloway's lab in a mate of *Ps. aeruginosa* PAT with PAO; Holloway and Jennings, 1958; Stanisich and Holloway, 1969) was mated with J1. (PTO13 was the generous gift of Dr. Eric Moody at the University of Texas Health Science Center, San Antonio, Texas.) There was a strong possibility that other genes from PTO13 were also present in JPT3, the new isolate. But, important as this later became to interpreting oxygen monitor and spectrophotometric enzyme activity data, no effective control could be exerted to prevent it. The use of a short mating time prior to selection of an $FP2^+$ *PMOcre*$^+$ exconjugant

FIGURE 21

COMPARISON OF PROTOCATECHUATE RING-FISSION PEAKS GENERATED BY JPT3-4 AND JPT8N-80 EXTRACTS



JPT8N-80 extract activity (lower left) was detectable in extracts suspended in 50mM phosphate buffer (Hegeman, 1966) with 1 mM EDTA.

JPT3-4 extract activity (upper left) was detectable only when MgEDTA was used with the phosphate buffer.

Note the comparison of the UV absorbance spectra obtained with "authentic" PROCAT used in this study, with those of para-hydroxybenzoic acid and para-hydroxybenzaldehyde, which are biological precursors of PROCAT in the proposed pathway.

Both organisms seem to show an activity that is removing compounds absorbing at 247 and 285 nm, creating a component which absorbs at an intermediate wavelength with approximately the same extinction coefficient.

was attempted, but no plasmid-bearing colonies were detected.

In exconjugants from JPT3-4 $FP2^+$ x JPT8N-80 F^- mates, the time for appearance of all these varied types was T+105 minutes. The abrupt appearance of apparent recombinants well into a mate indicates that there is some chromosomal involvement in genes being selected by the selective media. In this case, the media was M100-Neomycin 400 ppm. JPT8N-80 controls would not colonize this media. Small translucent "pinpoint" colonies (J3-8R1C, D, and F) were subsequently found to be devoid of the JPT3-4 plasmid band (see Table 3 and Figure 19). J3-8R1A, B, and E were much better growers, and were also subsequently determined to possess the JPT3-4 plasmid. These strains were most like JPT3-4 in their induction patterns, but retained JPT8N-80's lack of green pigment production on rich media.

JPT3-4 $FP2^+$ x PAS102N F^- mates also produced exconjugants possessing a range of activities. As can be seen from Table 3 , those falling into Groups I, III, and IV all possessed the JPT3-4-type plasmid, and all expressed an inducible para-cresol hydroxylase and 4MCAT activity, as well as PROCAT activity. With three exceptions (J3-102B, G, and H), they also possessed hydroxylase activities for meta- and ortho-cresol and phenol. Of the three singled out, only J3-102B was tested in the oxygen monitor, which showed that the meta-, ortho-cresol and phenol hydroxylase was

clearly there, inducible at levels far greater than the recipient organism, PAS102N, had ever shown, but that the level of induction was not as high as in parental JPT3-4. Since all of the J3-102s were, like PAS102N, auxotrophic for threonine, and actually did not grow well on a cresol substrate, even when supplemented with 40 ppm threonine, their growth had been in threonine-supplemented succinate broth, followed by a two-hour induction exposure to the inducer phenolic. Possibly succinate inhibited the full induction of hydroxylating activities in some recombinants.

Putative recombinants from the PAS102N recipients had occurred after 60 minutes of mating. This, too, indicated some linkage of a critical gene to the JPT3-4 chromosome. No colonies possessing the JPT3-4 plasmid could be found in recipient-selective Luria-Neomycin plates taken earlier in the mates, though only a dozen colonies were tested out of 150 isolated, hence this finding is not as significant as it could be. Most of the exconjugants on recombinant selective plates did possess the plasmid; all colonies shown to oxidize the catechols possessed a plasmid. Colonies without a plasmid invariably lacked meta-, ortho-cresol and phenol hydroxylase activities and catechol 2,3-oxygenase. Surprisingly, all J3-102 exconjugants--even those not bearing the JPT3-4 plasmid--showed the para-cresol/4MCAT/PROCAT oxidation pattern. This was clear evidence of a separate

site for genes coding for para-cresol degradation via PROCAT, a site which must either be chromosomal, or must lie on a plasmid separate from the JPT3-4 FP-like plasmid.

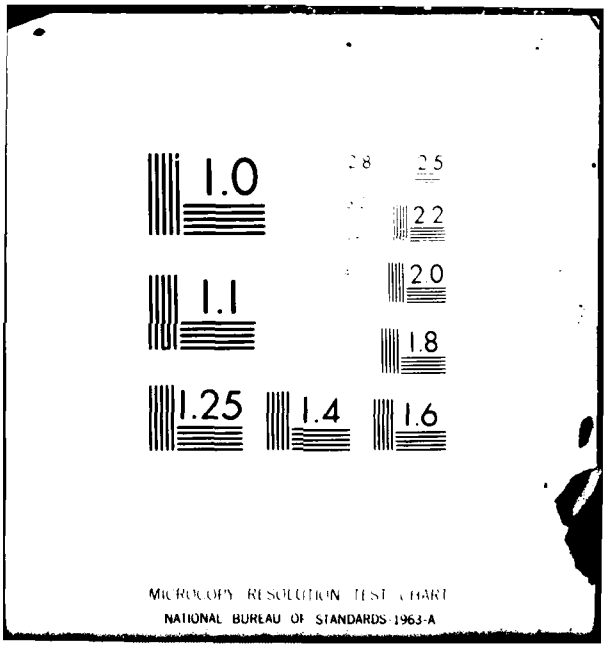
Interpretation of the significance of the ubiquitous presence of the FP-like plasmid in exconjugants with the para-, meta-, ortho-cresol, phenol HX/2,30X pattern is difficult. PT013 had been shown not to possess the ability to grow on cresols as sole carbon source at concentrations of 500 ppm (Egan, unpublished data). There is no evidence from the literature that FP2 codes any degradative functions. Therefore, its presence in *PMOcre*$^{+}\emptyset^{+}$ recombinants may reflect a relatively recent genetic incorporation of data from JPT3-4; or, more likely, may reflect a high probability of its transfer in any conjugation event of sufficient duration.

FP2 has been shown to be present in more than one copy per cell (Stanisich and Holloway, 1972), and to rapidly spread to about 60% of a properly receptive population during log growth. Less than one doubling time was allowed during the above mates, hence the spread of FP2 would not be too great. But its presence in most of the putative recombinants must be significant. The mechanism by which FP2 promotes the transfer of chromosomal genes is one of integration at a specific site into the host chromosome (Holloway *et al.*, 1971), followed by a transfer "push." Chromosomal marker transfer is not required for FP2's

infectious communication. However, conversely, it has been shown that 30 to 80% of all chromosomal transcipients inherit FP2 (Loutit *et al.*, 1968; Stanisich and Holloway, 1969; Pemberton, 1971; Pemberton and Clark, 1973). Holloway (1975) concluded that a chromosomally integrated FP2 mobilizes the chromosome in transfers, and that a separate FP2 is independently transferred in a per centage of the mates which last long enough for some chromosomal transfer. Thus, the presence of FP2 in all transcipient J3-102s having the 2,30X activities may reflect the late chromosomal location of such information, or of information critical to its expression.

AD-A117 730 TRINITY UNIV SAN ANTONIO TEX F/G 6/13
GENETIC ANALYSIS OF THE NATURE OF GENES CODING EARLY ENZYMES IN--ETC(U)
APR 82 H D COBB, J W EGAN, W E OLIVE F49620-79-C-0192
UNCLASSIFIED AFOSR-TR-82-0615 NL

END
DATE FILMED
08-82
DTIC


1.0
2.8
2.5
2.2
2.0
1.1
1.8
1.25
1.4
1.6
MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

CONCLUSIONS

In attempting to fulfill the specific aims of this project, the following tasks have been accomplished:

(1) The primary enzyme pathways for *Pseudomonas aeruginosa* JPT3-4 and JPT8N-80, during their growth on phenol, the three cresol isomers (para-, meta-, and ortho-cresol), and benzoate, have been determined. Some ambiguities in interpretation of data remain; e.g., the nature of the PROCAT oxygenase in JPT3-4, and the nature of the apparent 1,2-fissioning of catechols in JPT8N.

(2) A comparison has been made of baseline induction data for donors, recipients, and putative recombinants, using the donor strain, JPT3-4, and the two recipient strains, JPT8N-80 and PAS102N.

(3) The plasmid populations of the organisms involved have been visualized via the best available electrophoretic techniques, and interpreted for relationship to cresol metabolism behaviour in the test organisms.

Based on these findings, the following conclusions can be drawn:

1. Information for para-cresol methylhydroxylase is linked to 4-methyl-catechol and protocatechuate activity, and is probably chromosomally located prior to 60 min. on the JPT3-4 chromosome.

2. JPT3-4, when bearing the FP2 sex factor, can successfully transfer all of its phenolic degradative genes to cresol-incompetent recipients. Such transfer is time-dependent, appears to involve genes late on the JPT3-4 chromosome, and results in a very high per centage of transcipients bearing the FP2 plasmid.

3. JPT3-4 carries a specific hydroxylating activity for para-cresol, probably a methylhydroxylase; a less specific ring-hydroxylase acting on para-, meta-, and ortho-cresol and phenol; a parahydroxybenzoic acid-specific ring hydroxylase; a protocatechuate oxygenase; and a 2,3-oxygenase activity acting on the catechol derivatives of the three cresol isomers; a benzoate oxidase and catechol 1,2-oxygenase.

4. JPT8N carries a similarly-acting para-cresol methylhydroxylase, parahydroxybenzoic acid ring hydroxylase, and protocatechuate oxygenase that may be different from that carried by JPT3-4 in that no cofactor appears to be required for its action; as well as a benzoate oxidase, and 1,2-oxygenases acting on the catechols.

5. PAS102N carries no effective phenolic degradative enzymes, but can express such genes transferred in from JPT3-4 $FP2^{+}$.

Findings that FP2 is involved in phenolic degradative gene transfer are consistent with any of the following interpretations:

1. FP2 may be mobilizing a non-self-transmissible gene in JPT3-4--one undetected in DNA extracts--coding for 2,3-oxygenase (and perhaps later) enzymes in the "meta" ring cleavage pathway for the catechols, and perhaps coding for a nonspecific cresol ring hydroxylase.

2. JPT3-4 may represent a clone in which FP2 has integrated an excised sequence from the original J1 chromosome coding "meta" cleavage genes, and possibly the nonspecific cresol ring hydroxylase.

3. All cresol degradative genes (and required ancillary genes) are chromosomally coded in JPT3-4, and may be mobilized by the FP2 sex factor, which is coinherited by chromosomal transcipients at high frequency.

REFERENCES

Aaij, C., and P. Borst. 1972. The gel electrophoresis of DNA. Biochem. Biophys. Acta. 269:197-200.

Barker, A. N. 1949. Some microbiological aspects of sewage purification. J. Inst. Serv. Purif. 1:7-22.

Bauer, W., and J. Vinograd. 1968. The interaction of closed circular DNA with intercalative dyes. I. The superhelix density of SV40 DNA in the presence and absence of dye. J. Mol. Biol. 33:141-171.

Bayly, R. C., S. Dagley, and D. T. Gibson. 1966. The metabolism of cresol by species of *Pseudomonas*. Biochem. J. 101:293-301.

Bayly, R. C., and G. J. Wigmore. 1973. Metabolism of phenol and cresol by mutants of *Pseudomonas putida*. J. Bacteriol. 113:1112-1120.

Behn, V. C., and P. Monadjemi. 1968. Developments in biological filtration. *In* Advances in Water Quality Improvement, vol. 1. E. F. Gloyna and W. W. Eckenfelder (eds). University of Texas Press, Austin, pp. 204-214.

Boyer, J. W. 1974. Restriction and modification of DNA; enzyme substrates. Fed. Proc. 33:1125-1127.

Brebion, G., S. Prat, and J. Prat. 1963. A study of purification by biological means, involving the successive actions of selected and adapted strains of microorganisms for the treatment of residual trate wastes from paper pulp factories. J. of Air and Water Poll. 7:413-425.

Brewer, W. E., D. Bird, M. A. Struck, Jr., E. W. Poth, Jr., and W. W. Melvin, Jr. 1968. Toxicity of untreated Kelly AFB sewage to fathead minnows (*Pimephales promelas*). USAF Environmental Health Lab Technical Report REHL (K) 68-20.

Bushnell, L. D., and H. F. Haas. 1941. The utilization of certain hydrocarbons by microorganisms. J. Bacteriol. 41:653-675.

Cavalli, L. L., J. Lederberg, and E. M. Lederberg. 1953. An infective factor controlling sex compatibility in *Bacterium coli*. J. Gen. Microbiol. 8:89-102.

Chakrabarty, A. M. 1972. Genetic basis for the biodegradation of salicylate in *Pseudomonas*. J. Bacteriol. 112:815-823.

Chakrabarty, A. M. 1974. Dissociation of a degradative plasmid aggregate in *Pseudomonas*. J. Bacteriol. 118:815-820.

Chakrabarty, A. M., G. Chow, and I. C. Gunsalus. 1973. Genetic regulation of octane dissimilation plasmid in *Pseudomonas*. Proc. Nat. Acad. Sci. U.S.A. 70:1137-1140.

Chakrabarty, A. M., and I. C. Gunsalus. 1969. Defective phage and chromosome mobilization in *Pseudomonas putida*. Proc. Nat. Acad. Sci. U.S.A. 64:1217-1223.

Chapman, P. J. and D. J. Hopper. 1968. The bacterial metabolism of 2,4-xylenol. Biochem. J. 110:491-498.

Clark, A. J., and G. J. Warren. 1979. Conjugal transmission of plasmids. Annu. Rev. Genet. 13:99-125.

Clewell, D. B., and D. R. Helinski. 1969. Super-coiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular form. Proc. Nat. Acad. Sci. U.S.A. 62:1159-1166.

Cobb, H. D., Jr., W. E. Olive, Jr., J. W. Egan and D. J. Hansen. 1979. Biodegradation of phenolic paint-stripping waste: Laboratory evaluation of a fixed film batch reactor. (Trinity University.) AFESC, Engineering and Services Laboratory, ESL-TR-79-11, Tyndall AFB, Florida 32403.

Cohen, S. N. and C. A. Miller. 1969. Multiple molecular species of circular R-factor DNA isolated from *Escherichia coli*. Nature (London) 224:1273-1277.

Dagley, S. and M. D. Patel. 1957. Oxidation of p-cresol and related compounds by a *Pseudomonas*. Biochem. J. 66: 227-233.

Duggleby, C. J., S. A. Bayley, M. J. Worsey, P. A. Williams, and P. Broda. 1977. Molecular sizes and relationships of TOL plasmids in *Pseudomonas*. J. Bacteriol. 130: 1274-1280.

Dunn, N. W., and I. C. Gunsalus. 1973. Transmissible plasmid coding early enzymes of naphthalene oxidation in *Pseudomonas putida*. J. Bacteriol. 114:974-979.

Falkow, S., J. A. Wohleiter, R. V. Citarella, and L. S. Baron. 1964. Transfer of episomic elements to *Proteus*. I. Transfer of F-linked chromosomal determinants. J. Bacteriol. 87:209-219.

Falkow, S., P. Guerry, R. W. Hedges, and N. Datta. 1975. Polynucleotide sequence relationships among plasmids of the I compatibility complex. J. Gen. Microbiol. 85:65-76.

Feist, C. F. and G. D. Hegeman. 1969. Phenol and benzoate metabolism by *Pseudomonas putida:* regulation of tangential pathways. J. Bacteriol. 100:869-877.

Fishburn, C. A., R. A. Callahan, A. M. Elliott, and W. M. Melvin, Jr. 1970. Biotreatability and toxicity of selected phenolic paint strippers. USAF Environmental Health Laboratory (AFLC) Technical Report, EHL(K) 70-22.

Freifelder, D. R., and D. Freifelder. 1968. Studies on *Escherichia coli* sex factors. I. Specific labeling of F'lac DNA. J. Mol. Biol. 32:15-23.

Friello, D. A., J. R. Mylroie, D. T. Gibson, J. E. Rogers, and A. M. Chakrabarty. 1976. XYL, a nonconjugative xylene degradative plasmid in *Pseudomonas Pxy*. J. Bacteriol. 127:1217-1224.

Fujiwara, M., L. A. Golovlera, Y. Sacki, M. Nozaki, and O. Hayaishi. 1975. Extradiol cleavage of 3-substituted catechols by an intradiol dioxygenase, pyrocatechase, from a *Pseudomonad*. J. Biol. Chem. 250:4848-4855.

Greene, P. J., M. G. Betlach, H. W. Boyer, and H. M. Goodman. 1974. The EcoRI restriction endonuclease, pp. 87-105. *In* R. B. Wickner (ed.), Methods in Molecular Biology, vol.7. *DNA Replication*. Marcel Dekker Inc., N.Y.

Guerry, P., D. J. LeBlanc, and S. Falkow. 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. 116:1064-1066.

Gunsalus, I. C., C. A. Tyson, R. L. Tsai, and J. D. Lipscomb. 1971.

Hansen, J. B. and R. H. Olsen. 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol. 135:227-238.

Hatcher, A. B. 1977. The metabolism of cresols by *Pseudomonas* J1. Masters Thesis. Trinity University, San Antonio, Texas, 70pp.

Hawkes, H. A. 1961. An ecological approach to some bacteria bed problems. J. Inst. Serv.Purif. 2:105-132.

Hawkes, H. A. 1963. The Ecology of Waste Water Treatment. Permagon Press, The MacMillan Company, N.Y.

Hayaishi, O. 1966. Crystalline oxygenases of pseudomonads. Bacteriol. Rev. 30:720-731.

Hayaishi, O., H. Taniuchi, and J. Kojima. 1962. Reversible inactivation of oxygenases by oxygen. Federation Proc. 21:52. (Abstr.)

Hegeman, G. D. 1966. Synthesis of enzymes of the mandelate pathway by *Pseudomonas putida*. I. Synthesis of the enzymes by the wild type. J. Bacteriol. 91:1140-1154.

Helling, R. B. 1975. Eukaryotic genes in prokaryotic cells. Stadler Symp. 7:15-36.

Helmers, E. N., J. D. Frame, A. E. Greenburg, and C. N. Sawyer. 1952. Nutritional requirements in the biological stabilization of industrial wastes; III--Treatment with supplementary nutrients. Sewage Industr. Wastes, 24: 496-507.

Hewetson, L., H. M. Dunn and N. W. Dunn. 1978. Evidence for a transmissible catabolic plasmid in *Pseudomonas putida* encoding the degradation of p-cresol via the protocatechuate *ortho*-cleavage pathway. Genet. Res. 32:249-255.

Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26:365-369.

Holloway, B. W. 1955. Genetic recombination in *Pseudomonas aeruginosa*. Gen. Microbiol. 13:572-581.

Holloway, B. W. 1956. Self-fertility in *Pseudomonas aeruginosa*. J. Gen. Microbiol. 15:221-224.

Holloway, B. W. 1969. Genetics of *Pseudomonas*. Bacteriol. Rev. 33:419-433.

Holloway, B. W. 1975. Genetic Organization of *Pseudomonas*, pp. 133-161. *In* P. Clarke and M. Richmond (ed.), Genetics and Biochemistry of *Pseudomonas*. John Wiley and Sons, Inc., New York.

Holloway, B. W., and P. A. Jennings. 1958. An infectious fertility factor for *Pseudomonas aeruginosa*. Nature 181:855-856.

Holloway, B. W., V. Krishnapillai, and V. A. Stanisich. 1971. *Pseudomonas* genetics. Ann. Rev. Genet. 5:425-560.

Hopper, D. J. 1976. The Hydroxylation of *p*-cresol and its conversion to *p*-hydroxybenzaldehyde in *Pseudomanas putida*. Biochem. Biophys. Res. Commun. 69:462-468.

Hopper, D. J. And D. G. Taylor. 1975. Pathways for the degradation of m-cresol and p-cresol by *Pseudomonas putida*. J. Bacteriol. 122:1-6.

Hopper, D. J., and D. G. Taylor. 1977. The purification and properties of *p*-cresol-(acceptor) oxidoreductase (hydroxylating), a flavocytochrome from *Ps. putida*. Biochem. J. 167:155-162.

Hosokawa, K., and R.Y. Stanier. 1966.

Huber, L. 1967. Disposal of effluents from petroleum refineries and petrochemical plants. 22nd Annual Purdue Industrial Waste Conference.

Humphreys, G. O., G. A. Willshaw and E. S. Anderson. 1975. A simple method for the preparation of large quantities of pure plasmid DNA. Biochem. Biophys. Acta 383:457-483.

Ingold, R. S. 1940. Oxidation-reduction enzymes in activated sludge. Sewage Wks. J. 12:862-874.

Jacob, F. 1966. Genetics of the bacterial cell. Science 152:1470-1478.

Jacob, F., P. Schaeffer and E. L. Wollman. 1960. Episomic elements in bacteria, pp. 67-91. *In* W. Hayes and R. Clowes (eds.), Microbial Genetics. 10th Symp. Soc. Gen. Microbiol. Cambridge University Press, Cambridge.

Jacob, F., and E. L. Wollman. 1961. Sexuality and the Genetics of Bacteria. Academic Press Inc., New York.

Keat, M. J. and D.J. Hopper. 1978. The aromatic alcohol dehydrogenases in *Pseudomonas putida*. N.C.I.B. 9869 grown on 3,5-xylenol and p-cresol. Biochem. J. 175: 659-667.

Kostenbader, P. D., and J. W. Flecksteiner. 1969. Biological oxidation of coke plant weak ammonia liquor. J. Water Poll. Control Fed. 41:(2).

Kroop, R. H. 1973. Treatment of phenolic aircraft paint stripping wastewater. Proceedings of the 28th Annual Purdue Industrial Waste Conference, Part 2, p. 68.

Lanouette, K. H. 1977. Treatment of phenolic waste water. Chemical Engineering Deskbook, October, pp.99-106.

Lederberg, J. and E. L. Tatum. 1946. Gene recombination in *Escherichia coli*. Nature, London. 158:558.

Lederberg, J. and E. M. Lederberg. 1952. Replica plating and indirect selection of bacterial mutants. J. Bacteriol. 63:399-406.

Lennox, E. S. 1955. Transduction of linked genetic characters of the host by Bacteriophage P1. Virology 1:190-206.

Lode, E. T. and M. J. Coon. 1971.

Lopez-Alvarez, J., C. L. Gyles, P. L. Shipley, and S. Falkow. 1980. Genetics and molecular characterization of virulent plasmids of bovine septicemic *E. coli*. J. Bacteriol. 141:758-769.

Lopez, P., M. Espinosa, M. Piechowska, and D. Shugar. 1980. Influence of bacteriophage PBS1 and ϕW-14 deoxyribonucleic acids on homologous deoxyribonucleic acid uptake and transformation in competent *Bacillus subtilis*. J. Bacteriol. 143:50-58.

Loutit, J. S., and L. E. Pearce, and M. G. Marinus. 1968. Investigation of the mating system of *Pseudomonas aeruginosa* strain 1. I. Kinetic studies. Genet. Res. 12:29-36.

MacDonald, D. L., R. Y. Stanier, and J. L. Ingraham. 1954.

Metcalf and Eddy, Inc. 1972. Wastewater Engineering: Collection, Treatment, Disposal. (McGraw-Hill series in water resources and environmental engineering.) P. 377. McGraw-Hill. New York.

Meyers, J. C., D. Sanchez, L. P. Elwell, and S. Falkow. 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. 127:1529-1537.

Mitchell, C. D. 1974. An ecological approach to the biological oxidation of cresylic acid, Phase I; isolation and identification of the bacteria involved in the degradation of cresol. Masters' Thesis, Trinity University, San Antonio, Texas.

Nakashio, M. 1969. Phenolic waste water treatment by activated sludge process. J. Ferment. Technol. 47:389-393.

Ohta Y., I. J. Higgins, and D. W. Ribbons. 1975. Metabolism of resorcinylic compounds by bacteria. Purification and properties of orcinol hydroxylase from *Ps. putida* 01. J. Biol. Chem. 250: 3814-3825.

Olive, W. E., Jr. 1975. An ecological approach to the biological treatment of cresylic acid laden waste water. Master's Thesis. Trinity University, San Antonio (May).

Olive, W. E., Jr., H. D. Cobb, Jr., and R. M. Atherton. 1976. Biological treatment of cresylic acid laden waste water, pp. 381-388. *In* J. M. Sharpley and A. M. Kaplan (eds.), Proceedings of the Third International Biodegradation Symposium. Applied Science Publishers, Inc. London.

Ornston, L. N. 1966a. The conversion of catechol and protocatechuate to beta-ketoadipate by *Pseudomonas putida*. II. Enzymes of the catechol pathway. J. Biol. Chem. 241:3795-3799.

Ornston, L. N. 1966b. The conversion of catechol and protocatechuate to beta-ketoadipate by *Pseudomonas putida*. III. Enzymes of the catechol pathway. J. Biol. Chem. 241:3795-3799.

Ornston, L. N. 1966c. The conversion of catechol and protocatechuate to beta-ketoadipate by *Pseudomonas putida*. IV. Regulation. J. Biol. Chem. 241:3800-3810.

Ornston, L. N. 1971. Regulation of catabolic pathways in *Pseudomonas*. Bacteriol. Rev. 35:87-116.

Ornston, L. N. and R. Y. Stanier. 1966. The conversion of catechol and protocatechuate to beta-ketoadipate by *Pseudomonas putida*. I. Biochemistry. J. Biol. Chem. 241:3776-3786.

Palchaudhuri, S., and A. M. Chakrabarty. 1976. Isolation of plasmid deoxyribonucleic acid from *Pseudomonas putida*. J. Bacteriol. 126:410-416.

Pemberton, J. M., and A. J. Clark. 1973. Detection and characterization of plasmids in *Pseudomonas aeruginosa* strain PAO. J. Bacteriol. 114:424-433.

Pemberton, J. M., and B. W. Holloway. 1972. Chromosome mapping in *Pseudomonas aeruginosa*. Genet. Res. Camb. 19:251-260.

Perrotti, A. E. 1975. Activated carbon treatment of phenolic paint stripping wastewater. Facet Enterprises Industries, Inc., Final Report to Air Force Civil Engineering Center AFSC, AFCEC-TR-75-14.

Radhakrishnana, I., and A. K. S. Ray. 1971. Activated sludge studies with phenol bacteria. J. Water Poll. Control Fed. 43:(11).

Rao, I. P. S. 1969. Pilot plant studies on treatment of phenolic wastes by high-rate deep trickling filture. Environmental Health, 11:366.

Rheinwald, J. C., A. M. Chakrabarty, and I. C. Gunsalus. 1973. A transmissible plasmid controlling camphor oxidation in *Pseudomonas putida*. Proc. Nat. Acad. Sci. U.S.A. 70:855-889.

Ribbons, D. W. 1966. Metabolism of o-cresol by *Pseudomonas aeruginosa* strain T1. J. Gen. Microbiol. 44:221-231.

Ribbons, D. W. 1970. Specificity of monohydric phenol oxidation by *meta* cleavage pathways in *Pseudomonas aeruginosa* T1. Arch. Mikrobiol. 74:103-115.

Rolfe, B. and B. W. Holloway, 1966. Alterations in host specificity of bacterial deoxyribonucleic acid after an increase in growth temperature of *Pseudomonas aeruginosa*. J. Bacteriol. 92:43-48.

Rolfe, B. and B. W. Holloway. 1968. Genetic control of DNA specificity in *Pseudomonas aeruginosa*. Genet. Res. 12:94-102.

Sala-Trepat, J. M., K. Murray, and P. A. Williams. 1972. The metabolic divergence in the *meta* cleavage of catechols by *Pseudomonas putida* NCIB 10015. Eur. J. of Biochem. 28:347-356.

Sistrom, W. R., and R. Y. Stanier. 1954. The mechanism of formation of beta-ketoadipic acid by bacteria. J. Biol. Chem. 120:821-836.

Stanier, R. Y. 1947. Simultaneous adaptation: a new technique for the study of metabolic pathways. J. Bacteriol. 54:339-348.

Stanier, R. Y., and J. L. Ingraham. 1954.

Stanisich, V., and B. W. Holloway. 1969. Conjugation in *Pseudomonas aeruginosa*. Genetics 61:327-339.

Stanisich, V. A., and B. W. Holloway. 1972. A mutant sex factor of *Pseudomonas aeruginosa*. Genet. Res. 19:

Stanisich, V. A., and M. H. Richmond. 1975. Gene transfer in the genus *Pseudomonas*. *In* Clarke and M. H. Richmond (eds.), pp. 163-190. Genetics and biochemistry of Pseudomonas. John Wiley and Sons, New York.

Stanisich, V. A., and B. W. Holloway. 1977.

Taniuchi, H., Y. Kojima, F. Kanetsuna, H. Ochiai, and O. Hayaishi. 1962. The mechanism of the autoxidation of oxygenases. Biochem. Biophys. Res. Comm. 8:97-103.

Timmis, K., F. Cabello, and S. N. Cohen. 1974. Utilization of two distinct modes of replication by a hybrid plasmid constructed *in vitro* from separate replicons. Proc. Natl. Acad. Sci. U.S.A. 71:4556-4560.

Williams, P. A., and K. Murray. 1974. Metabolism of benzoates and the methylbenzoates by *Pseudomonas putida (arvilla)* mt-2: Evidence for the existence of a TOL plasmid. J. Bacteriol. 120:416-423.

Williams, P. A., and M. J. Worsey. 1976. Ubiquity of plasmids coding for toluene and xylene metabolism in soil bacteria: Evidence for the existence of new TOL plasmids. J. Bacteriol. 125:818-828.

White, F. F., and E. W. Nester. 1980. Hairy root: plasmid encodes virulence traits in *Agrobacterium rhizogenes*. J. Bacteriol. 141:1134-1141.

Wong, C. L., and N. W. Dunn. 1974. Transmissible plasmid coding for the degradation of benzoate and m-toluate in *Pseudomonas arvilla* mt-2. Genet. Res. 23:227-232.

Worsey, M. J., and P. A. Williams. 1977. Characterization of a spontaneously occurring mutant of the TOL 20 plasmid in *Pseudomonas putida* MT20: possible regulatory implications. J. Bacteriol. 130:149-158.

Wu, C. H., M. K. Ornston, and L. N. Ornston. 1972. Genetic control of enzyme induction in the β-ketoadipate pathway of *Pseudomonas putida:* Two-point crosses with a regulatory mutant strain. J. Bacteriol. 109:796-802.

Wurm, H. J. 1968. The treatment of phenolic wastes. Proc. 23d Ann. Purdue Indus. Waste Conf., Part 2, p. 1054. (West Lafayette, Indiana.)

Yamamoto, K. R., B. M. Alberts, R. Benzinger, L. Lawhorne, and G. Treiber. 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. Virology 40:734-744.

Zoon, K. C., M. Habersat, and J. J. Scocca. 1976. Synthesis of envelope polypeptides by *Haemophilus influenzae* during development of competence for genetic transformation. J. Bacteriol. 127:545-554.


DATE
FILMED
8-8