Aquatic Bacteria and Pesticide Degradation

by

Ramon J. Seidler

Water Resources Research Institute

Oregon State University

Corvallis, Oregon

WRRI-93

August 1984

AQUATIC BACTERIA AND PESTICIDE DEGRADATION

by

Ramon J. Seidler
Department of Microbiology, Oregon State University

Final Technical Completion Report
Project Number G864-03

Submitted to

United States Department of the Interior
Geological Survey
Reston, Virginia 22092

Project Sponsored by:

Water Resources Research Institute
Oregon State University
Corvallis, Oregon 97331

The research on which this report is based was financed in part by the United States Department of the Interior as authorized by the Water Research and Development Act of 1978 (P.L. 95-467).

WRRI-93 August 1984

ABSTRACT

Thirty bacteria which exhibit the ability to utilize the herbicide 2,4-dichlorophenoxy acetate (2,4D) have been isolated from aquatic habitats in western Oregon. Bacteria utilizing 2,4-D were obtained from two-thirds of the attempted enrichment trials. All but one of the isolates are gram-negative rods, oxidase positive, and aerobic. Those examined have lateral or subpolar flagella and have 61-67% guanine plus cytosine content in the chromosomal DNA. The cultures conform to bacteria of the genus Alcaligenes or Pseudomonas. Enrichment experiments revealed approximately 10-20 indigenous bacteria per 100 ml of water were capable of using 2,4-D when supplied at high concentrations of 150 µg/ml. About 800 or more bacteria per 100 ml of water can use 1-5 µg of 2,4-D per ml. Over 60% of the isolates contained detectable DNA plasmids with molecular weights in the range of 10-53 megadaltons (md). Physical characterization of one large plasmid designated pEML159, revealed many similarities with a plasmid obtained from an Alcaligenes species isolated in Australia. Attempts to transfer the genetic ability for utilizing 2,4-D between bacteria met with limited success. Only pEML159 was transmissable, and only to species closely related to the donor strain. No genetic transfer of 2,4-D utilizing ability into bacteria numerically common in aquatic habitats was detected. It is concluded that aquatic bacteria which utilize 2,4-D are widespread but are present in relatively low numbers. Some of these organisms have the ability to utilize well over 150 µg/ml of 2,4-D and can rapidly cleanse aquatic

environments containing much smaller levels of the pesticide. These bacteria are usually able to metabolize other chlorinated aromatics of the 2,4-D structural family.

FOREWORD

The Water Resources Research Institute, located on the Oregon State University campus, serves the State of Oregon. The Institute fosters, encourages and facilitates water resources research and education involving all aspects of the quality and quantity of water available for beneficial use. The Institute administers and coordinates statewide and regional programs of multidisciplinary research in water and related land resources. The Institute provides a necessary communications and coordination link between the agencies of local, state and federal government, as well as the private sector, and the broad research community at universities in the state on matters of water-related research. The Institute also coordinates the interdisciplinary program of graduate education in water resources at Oregon State University.

It is Institute policy to make availabe the results of significant water-related research conducted in Oregon's universities and colleges. The Institute neither endorses nor rejects the findings of the authors of such research. It does recommend careful consideration of the accumulated facts by those concerned with the solution of water-related problems.

ACKNOWLEDGEMENTS

The results of this research are due to the dedicated efforts of the following individuals: James Schulke, Greg Albeke, Mark Rasmussen, Ann Schwartz, Terry Riscoe, Katrina Jepsen, and Joe Haberman who were undergraduate student assistants, and the able assistance of Ms. Linda Frazier (research technician) and Dr. Penny Amy (postdoctoral research associate). We express special appreciation to Dr. J. M. Pemberton for making his 2,4-D metabolizing cultures available to us.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Man-made chemicals are a beneficial and necessary part of life in any modern society. Chemicals are used for agriculture, silviculture, forestry, and industrial manufacture of materials that we enjoy, depend on, and demand in our civilized society. Over 800,000,000 lbs of pesticide products enter our environment annually (1). Some 34 to 51 million metric tons of wastes generated annually in this country are considered hazardous (2). Some chemicals and waste materials can have deleterious effects on society and on all biological life. Examples related to toxic chemical use which have received much attention in the public media include PCB's, DDT, Agent Orange (2,4,5-T) and Kepone. All of these chemicals are resistant to biodegradation and are slowly metabolized by very few microbial species.

The magnitude of disposal problems and the fate of toxic substances when improperly disposed of are overwhelming. It has been estimated that there are 32,000 to 50,000 disposal sites in the U.S. which contain hazardous wastes. Some 1,200 to 2,000 of these sites pose significant risks to human health (2). Complaints of miscarriages, increased rates of cancer, birth defects, and other illnesses have been associated with applications of toxic substances to the environment and with chemicals leaking from the drums at disposal sites (2).

Xenobiotics (synthetic chemicals) are transported through aquatic ecosystems. This can occur when groundwater aquifers get contaminated at waste dump sites or when precipitation of sufficient intensity follows the application of pesticide and

carries the chemicals into receiving streams, or with agricultural irrigation, where runoff of pesticides readily contaminate the nearest drainage ditch or stream. This "transport" of xenobiotics through aquatic ecosystems is the mechanism by which nontarget organisms become exposed to these agents. If these chemicals are not rapidly decomposed by bacteria, the potential for a deleterious impact increases (3,4).

Degradation of toxic substances may result from biotic or abiotic activities. The latter include photooxidation and adsorption to particulates (3,4). However, the abiotic processes only contribute to partial degradation (never to mineralization); thus, the activities of the biotic components are much more significant. Microbial action on xenobiotics is the most important consideration in their elimination from soil and aquatic ecosystems. If they are slowly or infrequently metabolized (Kepone, PCB's, 2,4,5-T) their persistence is of paramount importance because of their cycling through the food chain and exposing nontarget life, including humans, to their deleterious activity.

Scientists have argued that one critical research need is to establish whether a toxic substance will undergo biodegradation and thus be eliminated quickly from the environment (5,6). This rapid biodegradation helps to insure that the intended target organism(s) (weeds, insects, etc.) are primarily affected. Given the number and diverse structural types of synthetic chemicals, the search for information on their fate (possible biodegradation) and toxicity should not be conducted on a chemical-by-chemical basis (5).

Progress will be made considering compounds on a structure/activity functional basis, and generalizations of their fate and toxicity can provide significant contributions to the important environmental problems that society and science are facing (4-6).

Many bacteria which metabolize "exotic" xenobiotics have been found to possess DNA plasmids which genetically specify the biodegradation capacity (7-9). Plasmids are small, circular elements of DNA found in the bacterial cell but which replicate independently of the bacterial chromosome. These genetic elements often have the ability to transfer, sometimes with high efficiencies, to other bacterial species, related as well as unrelated, and thereby propagate the unique metabolic capacity into new species. Plasmids are responsible for conferring the ability on bacteria for metabolizing mono- and dichlorobenzoates, 2,4,5-T, 2,4-dichloro-phenoxyacetic acid (2,4-D), PCBs and 4-chloro-2-methylphenoxyacetic acid (7-11). It has recently become apparent that the evolution and spread of these so-called pesticide-degrading plasmids (PDP) play a significant role in the occurrence of microbial populations which are able to metabolize various types of xenobiotics (9).

Fears and concerns of citizens over the use of chemicals in agriculture, forestry, and other industries and over the spread of toxic chemicals are not confined to one region of the country. Such fears continue to grow as man continues to struggle with old problems and newly emerging pests which include tussock and gypsy moths, fruit flies, and the control of weeds to increase food crop yields. In the Pacific Northwest, large amounts of 2,4-D, 3-amino-

1,2,4-trizole (Amitrol T), and carbaryl (Sevin) are used in the control of various pests. Knowledge of the role of microbes and DNA plasmids in the biodegradation of these pesticides is limited to that of the chlorinated phenols.

The present study was conducted to obtain information on the occurrence of bacteria in aquatic environments which degrade 2,4-D. In addition, we have determined that these bacteria contain DNA plasmids which code for 2,4-D degradation, and we have measured the size and determined the endonuclease restriction patterns of these plasmids. At least some DNA plasmid fragments generated by restriction endonucleases are of virtually identical molecular weight among bacteria isolated from Australia and from Oregon. The widespread occurrence of these bacteria probably assists in cleansing the aquatic environment of pesticide following 2,4-D use in natural ecosystems.

METHODS

Isolation of 2,4-D utilizing bacteria

Aquatic samples were collected and supplemented with 150 µg/ml (final concentration) of 2,4-D and incubated at 30°C for one week on a rotary shaker. Samples were periodically removed, centrifuged, and scanned in the ultraviolet range for absorbance of 2,4-D at 283 nm. Crude cultures exhibiting an absorbance reduction of about 0.5 optical density units or more were considered to contain 2,4-D metabolizing bacteria. A 0.5 ml inoculum from such cultures was transferred to a broth enrichment medium containing mineral salts and usually 150 µg of 2,4-D/ml (12). The composition of this enrichment medium (EM) is: K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; $FeCl_3$, 0.02 g; $(NH_4)_2SO_4$, 1 g; distilled water to 1 liter, adjusted to pH 7. The medium can be supplemented with Noble agar (10 g/l) when necessary. The chlorinated phenol derivatives were added from filter sterilized stock solutions to final concentrations in the range of 100-150 µg/ml. The cultures were incubated for one week, scanned, and an inoculum was transferred again into sterile EM broth. This procedure was usually repeated two additional times prior to streaking onto EM Noble agar containing 150-250 µg/ml of 2,4-D. Isolated colonies were restreaked for purity and retested as pure cultures for 2,4-D utilization. Stocks were stored at -80°C for future studies.

Characterization of 2,4-D degrading bacteria

Isolates were examined for cell morphology, gram stain, growth at various temperatures, and for other conventional biochemical properties using standard bacteriological tests (13). Isolates

were also inoculated into API 20E test strips for an estimation of 20 diagnostic test results (Analytab Products, Inc., Plainview, NY).

DNA extraction and mol % G+C determination by the thermal melting technique

Chromosomal DNA was extracted using standard procedures (14). DNA samples were melted in an automatic programmable Beckman DU-8 spectrophotometer. The midpoint of the thermal melting curves (T_m) in 0.1 x SSC buffer was established and compared with that of an Escherichia coli standard. Each test DNA was melted in triplicate. Mol % G+C was calculated by the equation of Mandel et al. (15).

Transfer of plasmids expressing 2,4-D utilization

Transmission of plasmid DNA from isolates expressing 2,4-D utilization was attempted by a variety of procedures and techniques (16-18). In brief, prospective donor strains were inoculated along with a 2,4-D cured, antibiotic resistant Alcaligenes strain JMP228 (8,9) into broth medium. After overnight incubation at 35°C, mixtures were plated onto a medium containing 2,4-D as a sole source of carbon and an antibiotic (Nalidixic acid; Nal) which inactivated the donor parent strain. Only transconjugants which gained the ability to utilize 2,4-D and were resistant to Nal would grow on the agar medium. In other trials, mating experiments were repeated on Millipore filters of 0.45 µm porosity, as described by Coplin (18). This procedure allows detection of plasmid transfers at lower frequencies than those detectable by the broth procedure (19). Other mating trials used E. coli HB101 as a potential recipient.

Electron microscopy

Electron microscopy was used to ascertain the nature of flagella arrangement and cell morphology of selected isolates of 2,4-D degrading bacteria. For these observations cells were grown up in salt free LB broth (16) diluted 1:5 with distilled water. Log phase cells exhibiting motility were harvested by centrifugation at 1,000 x g for 5 min. The soft cell pellet was raised following the addition of distilled water. Cell suspensions were then placed onto Formvar coated grids and air-dried. The cells were shadowed with chromium and viewed in a Philips EM300 electron microscope.

Detecting the metabolism of chlorinated aromatic compounds

The detection of 2,4-D and other structurally related compounds was accomplished by scanning the centrifuged cell cultures. Each chemical exhibits a unique peak wavelength of ultraviolet light absorbance and the peak height was found to be linear with the concentration of the chemical left in the medium. The limits of detection were about 1 µg/ml for each compound tested for utilization.

Utilization of each compound was studied by pregrowing various pure cultures either on 2,4-D or in a complex LB broth medium (20). Approximately 10^8 cells were inoculated into 50 ml of EM broth containing one of the test pesticides present at 50 µg/ml as a sole source of carbon and incubated with shaking at 30°C. Aliquots were removed at weekly intervals, the cells were centrifuged from suspension, and the supernatants were scanned in the ultraviolet region for the peak height of the test chemical. Cultures were incubated and assayed for up to 7 weeks.

RESULTS AND DISCUSSION

Figure 1 illustrates the time course disappearance of 2,4-D in an enrichment culture as measured by ultraviolet scans of culture medium. The scans, run in the 210-310 nm range, illustrate the peak absorbance of 2,4-D at 283 nm. The top bar illustrates the initial zero time scan, with 150 µg/ml of 2,4-D, the second after 5 days and the third after 7 days of incubation. There was an approximate 50% decrease in absorbance of 2,4-D concentration in 5 days and about a 95% reduction by day 7. The various metabolic intermediates of 2,4-D metabolism such as the corresponding 2,4-dichlorinated phenol and the chlorinated catechol also absorb in the ultraviolet region. However, no intermediates accumulated in the cultures indicating complete degradation of the aromatic ring structure. Pure cultures subsequently isolated from these enrichments multiply on 2,4-D providing further proof for the complete utilization of the pesticide.

Some 45 enrichments were initiated for attempts at isolating 2,4-D degrading bacteria from aquatic specimens collected in western Oregon. Bacteria possessing this activity were isolated from 30 samples (Table 1). Water specimens and EM broth containing 2,4-D as a sole source of carbon were incubated statically or on a rotary shaker. The shaken cultures exhibited activity sooner, typically 4-6 days after initial inoculation of the pesticide. In most, but not all cases, over 100 µg/ml of 2,4-D was consumed within the first 6 days following its addition to a water sample.

Several attempts at enumeration of 2,4-D degrading bacteria by a 9-flask most probable number (MPN) system were made using overnight settled, returned activated sludge as an inoculum. Three

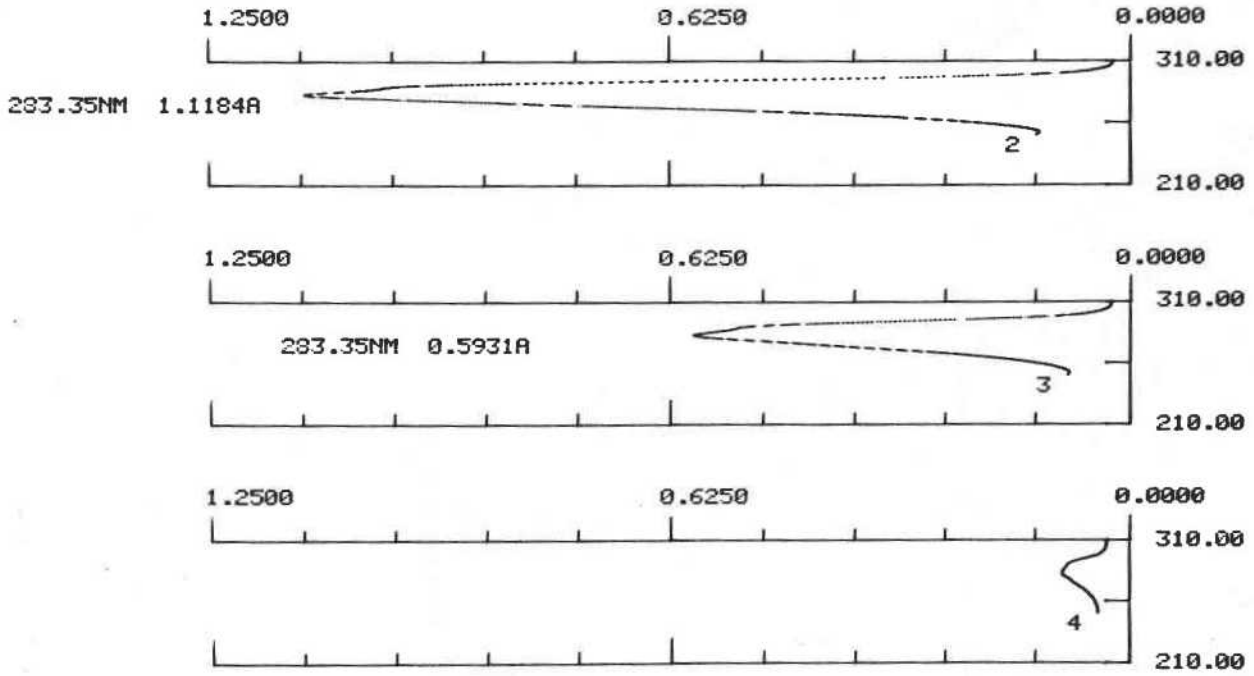

Figure 1. Time course illustrating the utilization of 2,4-D in an enrichment culture. Printout from the spectrophotometer for time zero (top bar), and for 5 and 7 days (middle and bottom bars, respectively) illustrating reduction in absorbance at 283 nm, the peak absorbance for 2,4-D.

Table 1. 2,4-D metabolizing bacteria and their source.

Isolate #	Source
EML130	Activated Sludge (static), Corvallis, OR
EML131	Activated Sludge (shaker), Corvallis, OR
EML132	Mary's River (static), Corvallis, OR
EML133	Mary's River (shaker), Corvallis, OR
EML134	Willamette River (static), Corvallis, OR
EML135	Willamette River (shaker), Corvallis, OR
EML137	Pond (shaker)
EML140	Mary's River (static), Philomath, OR
EML141	Mary's River (shaker), Philomath, OR
EML142	Pond (static), Philomath, OR
EML143	Pond (shaker), Philomath, OR
EML144	Pond (static), Corvallis, OR
EML145	Pond (shaker), Corvallis, OR
EML146	Willamette River, Corvallis, OR
EML147	Standing Water Near Willamette, Corvallis, OR
EML148	Juant Creek (sediment), Polk County, OR
EML149	Juant Creek (running water), Polk County, OR
EML155	Standing water, Gresham, OR
EML157	Return activated Sludge, Corvallis, OR
EML158	Willamette River, Corvallis, OR
EML159	Return activated Sludge, Corvallis, OR
EML160	Willamette River, Corvallis, OR
EWA	Willamette River
EWB	Willamette River
EWC	Willamette River
EWD	Willamette River
EMA	Mary's River
ERA	Return Activated Sludge
ERB	Return Activated Sludge
ERC	Return Activated Sludge

different volumes were inoculated in triplicate (30, 3, and 0.3 ml) into EM broth and incubated as enrichment flasks. A positive flask was scored when 25% or more of the 2,4-D had been consumed. When the flask initially contained 150 µg/ml of 2,4-D, the mean count for four trials was 13 organisms per 100 ml of clarified sewage. However, when a lower concentration of 2,4-D was used in the enrichments (1 µg/ml) and sequentially dosed such that a total of only 7 µg/ml of 2,4-D was added by the 9th day of incubation, the MPN exceeded 800 organisms/100 ml.

Cultures of 2,4-D degrading organisms were examined for purposes of identification. Overall, the isolates appeared rather homogeneous in their phenotypic properties. All but one was gram-negative. All were rod-shaped, catalase and oxidase positive, motile, and obligately aerobic. A flow chart summarizing the relationships among the gram-negative isolates based on the outcome of citrate utilization, growth at 40°C, and pigment production is illustrated in Table 2.

Representative cultures were selected for analysis of the mol % G+C content of chromosomal DNA (Table 3). The range of % G+C (61-67%) reflects species heterogeneity, i.e., all cultures may belong to one genus, but the genetic variability precludes that they all belong to a single species. The cell morphology of selected isolates illustrates rods with lateral or subpolar flagellar arrangement (Figures 2,3). The % G+C, flagellar arrangement, and phenotype are consistant with placement of the 2,4-D degrading bacteria in the genus Alcaligenes of Pseudomonas.

Table 2. Phenotypic differentiation of environmental isolates which degrade 2,4-D

+ Citrate Utilization			- Citrate Utilization
ERA	EML141	ERC	EML142
ERB	EML157	ERW	EML146
ERC	EML159	EML	EML148
EML130	JMP 228	EML137	EML155
EML134	EML147	EML140	EML160

+ Growth at 40°C			- Growth at 40°C		
EML141	EML130	EML134	JMP 228	EML132	EML137	EML155
ERC	EML147		ERA	EML146	EML140	EML160
EML157		EML159	ERB		EML142	EWC
EML158					EML148	EWD

+ pigment		- pigment		+ pigment		- pigment	
EML141 (ure+)	EML130	EML134	JMP 228 (ure+)	EML132	ERC	EML142
(onpg+)	EML147	EML158	ERA	EML146 (onpg+)	ERD	EML155 (onpg+)
	EML157(ure+)		ERB		137	
	ERC		EML159 (onpg+)		140	
					148	
					160	

Table 3. Mol % G+C content of chromosomal DNA from selected 2,4-D metabolizing isolates.

Isolate	% G + C
JMP 228	62.7 ± 0.3
JMP 134	63.7 ± 0.7
EWC	61.6 ± 0.4
ERC	63.7 ± 0.7
ERA	66.9 ± 0.7
EML155	62.1 ± 0.8
EWA	62.7 ± 0.3
EML157	62.9 ± 0.2
EML158	64.6 ± 0.2
EML159	61.1 ± 0.3

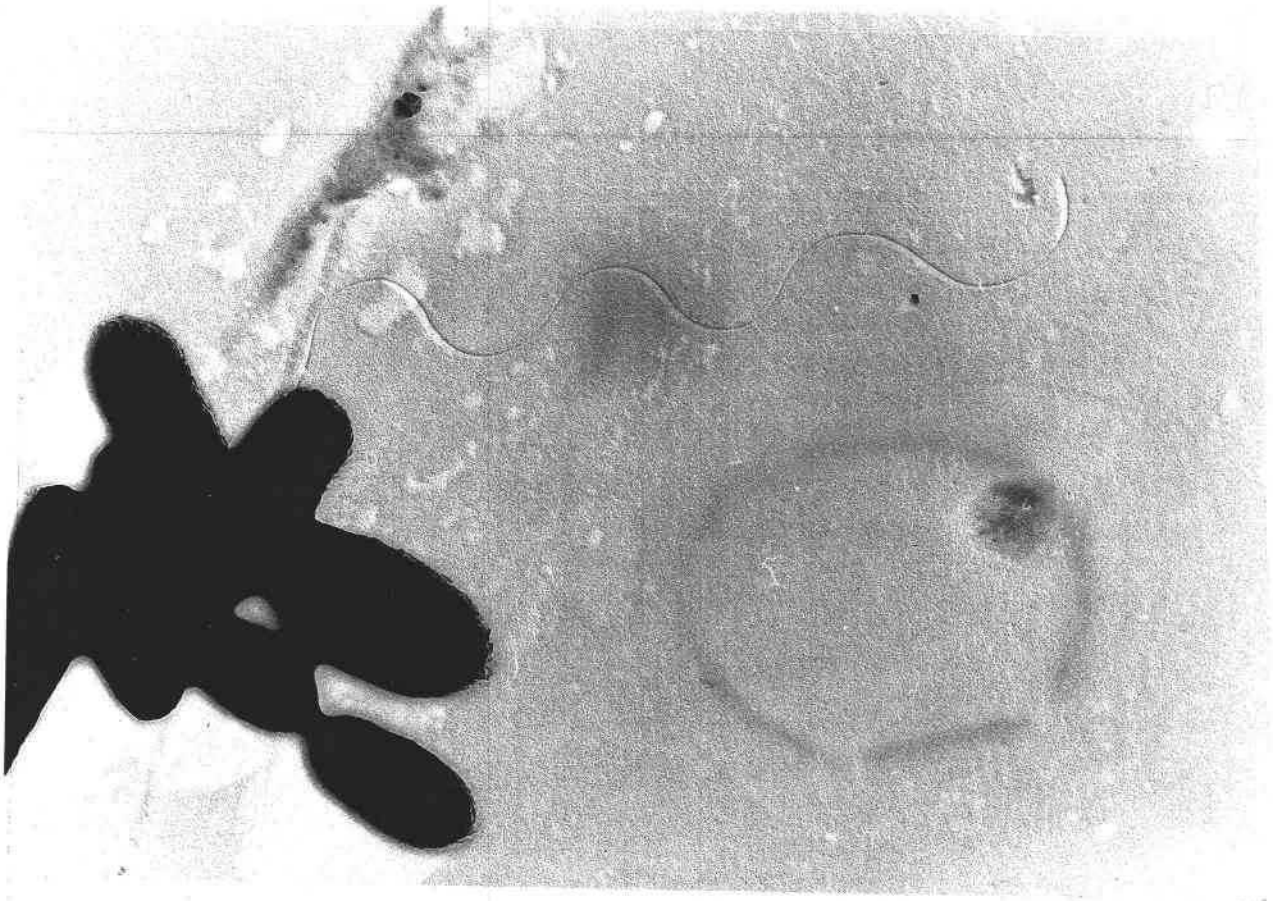

Figure 2. Electron micrograph illustrating culture of shadowcast ERC, showing subpolar insertion of flagella, typical of *Alcaligenes* species. X-18,000.

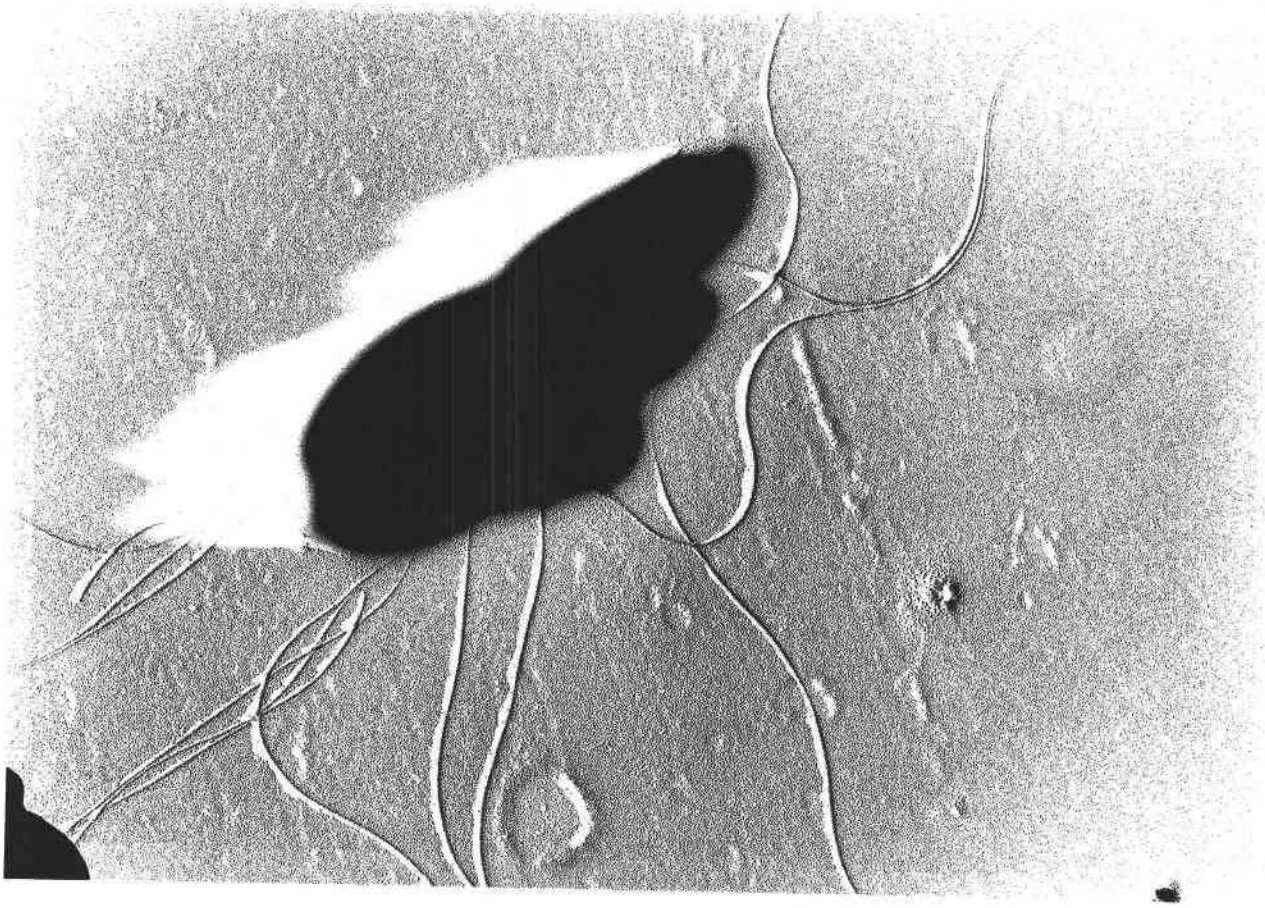

Figure 3. Electron micrograph illustrating culture of shadowcast EML158 with lateral insertion of flagella, typical of *Alcaligenes* species X-18,000.

Fourteen of 23 cultures examined contain detectable plasmids. Two molecular classes of plasmids have been detected, those of about 10 megadaltons (md) and a large size group of approximately 50-100 md. Figure 4 illustrates selected plasmids from the isolates. Gel lanes A, C, E, and J contain molecular weight markers from a Hind III digest of lambda phage DNA while lane L contains 8 molecular weight markers in E. coli V517.

The largest (and highest) molecular weight marker for V517 is 35.8 md and most plasmids from the 2,4-D utilizers are considerably larger. Cultures 141 (lane D), 148 (lane F), 146 (lane G), 155 (lane H), 159 (lane I), and 397 (lane K) appear to have plasmids of similar sizes. Some preparations as seen in lanes H, I, and L have a faint lower band which we believe represents contaminating chromosomal DNA. In other lanes (D and G) the position and sharpness of the additional band indicate additional plasmid molecules. These may be either different plasmid species or perhaps nicked or opened plasmid of the same species as present in the upper band.

Plasmids of similar sizes from strains EML159 and JMP397 (lanes I and K, respectively) were chosen for further comparisons. JMP397 contains a plasmid from a 2,4-D degrading Alcaligenes isolated in Australia (8,9), while culture EML159 was isolated from activated sludge from the Corvallis wastewater plant. Thermal denaturation curves of both plasmids revealed that the midpoints of the melting curves were virtually identical and occurred at 79°C in 0.1X strength SSC buffer (Fig. 5). This result reflects great similarities in their mol % G+C content. In order

DNA Plasmids from Selected 2,4-D Metabolizing Bacteria

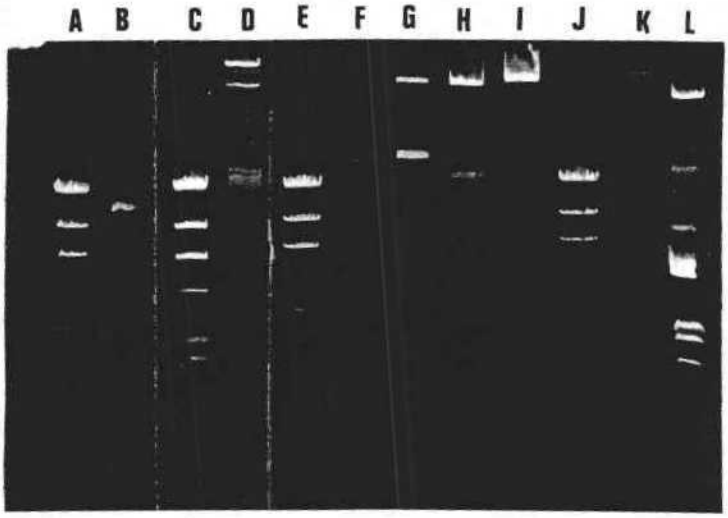

Figure 4. Photograph of an agarose gel illustrating selected plasmids isolated from 2,4-D utilizing bacteria.

Lane	Description
A,C,E,J,	Molecular weight standard prepared from a Hind III digest of Lambda phage
B	Plasmid from culture ERC of about 9.9. md size
D,F,G,H,I,K	Plasmid bands from cultures EML141, EML148, EML146, EML155, EML159, and JMP397, respectively. These plasmids are about 53 md size
L	Eight plasmids of known molecular weights present in *E. coli* V517

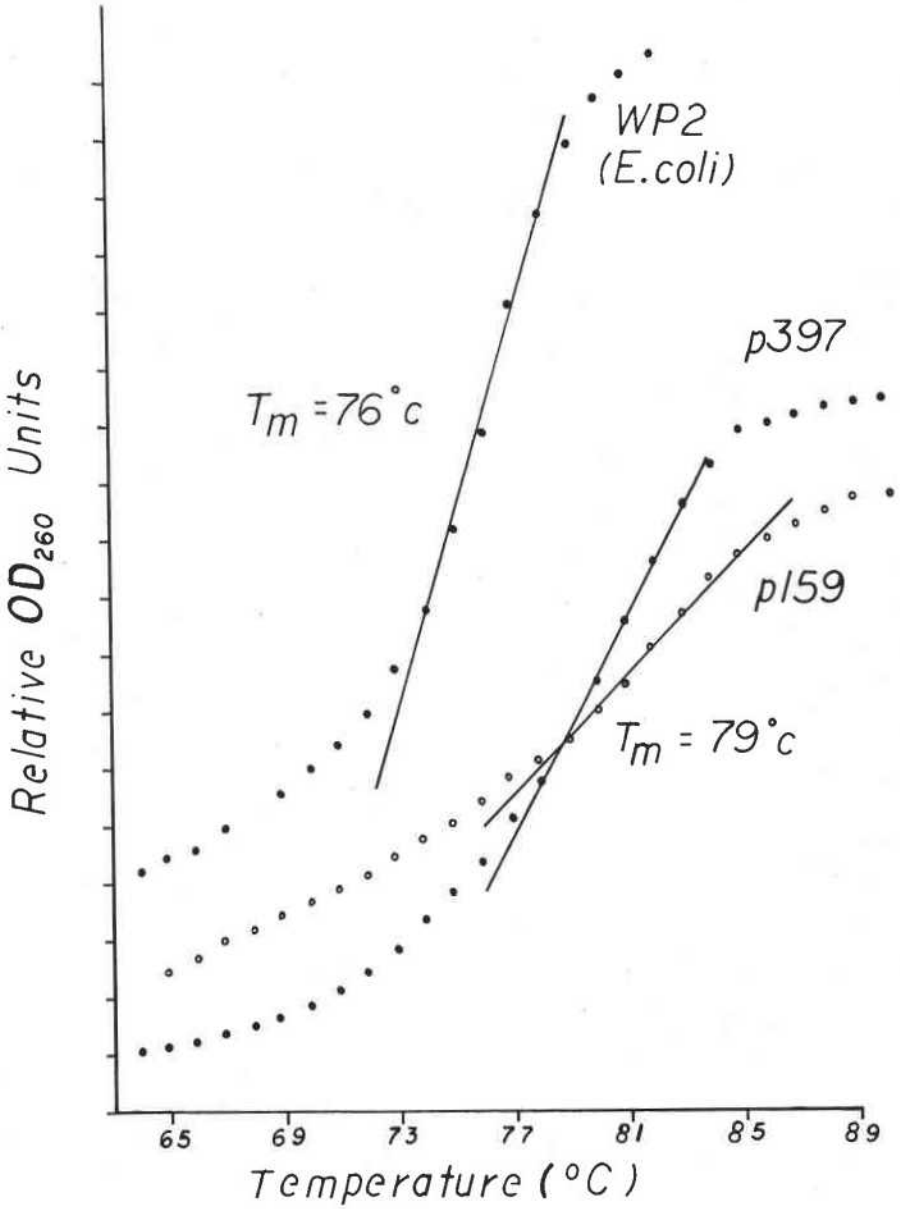

Figure 5. Thermal melting profiles for E. coli WP2 standard chromosomal DNA (T_m = 76.0°C), pEML159 (T_m = 79.0°C), and pJMP397 (T_m = 79.0°C). Both plasmids contain the same mol % G+C content of about 57%.

to examine whether these two plasmids in bacteria isolated on different continents contained further similarities, both plasmids were exposed to restriction endonuclease enzymes. The patterns of fragments generated revealed both differences and similarities in molecular weights (Table 4 and Fig. 6).

In Figure 6, lanes A, C, and E contain pEML159 while lanes B, D, and F contain pJMP397. Lanes A and B contain undigested plasmids (lower band lane A is contaminating chromosomal DNA). Both plasmids have migrated about the same distance and are therefore of similar molecular weight. Lanes C and D are the fragments produced by restriction enzyme Eco RI and lanes E and F illustrate fragments produced by enzyme Hind III. In each case more fragments are obvious from pJMP397. When one adds up the molecular weights of the plasmid fragments, the size of pEML159 is about 54.5 Md while that of pJMP397 is about 102.5 Md. Since both intact plasmids appear to comigrate, the molecular weights of the components should not differ by 50%. A similar difference in molecular weights occurs from the Hind III digestion (lanes E and F). Additional studies involving agarose gel electrophoresis of pJMP397 revealed that the preparations actually do contain two plasmids. The plasmids are 54.5 Md and 48 Md. The two plasmids are discerable in lane K of Figure 4.

A tabulation of fragment sizes generated from Eco RI digests of pEML159 and the two plasmids in culture JMP397 are presented in Table 4. Seven of the fragments from the JMP397 digest are identical in molecular weight to 7 of the fragments generated from pEML159. The other 6 fragments produced by the EcoRI digest represent those from the second plasmid of 54.5 md.

COMPARISON OF P159 AND P397

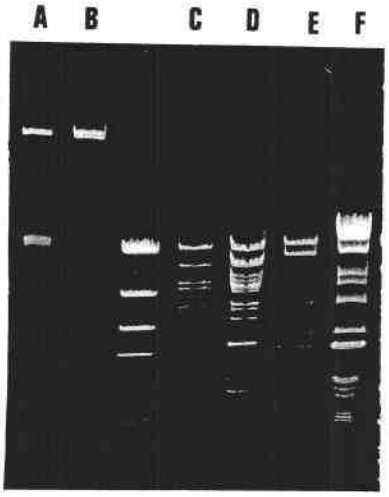

Figure 6. Agarose gel electrophoresis of pEML159 and pJMP397.

Lane	Description
A,B	Intact pEML159 and pJMP397, respectively
C,D	EcoRI digests of pEML159 and pJMP397, respectively
E,F	HindIII digests of pEML159 and pJMP397, respectively

Table 4. Molecular weights of DNA fragments generated from EcoRI digests of pEML159 and pJMP397.

	pEML159	pJMP397
	15.6 md	15.6 md
	12.5 md	12.5 md
	———	10.9 md
	9.4 md	9.4 md
	7.8 md	7.8 md
	5.7 md	5.7 md
	---	5.5,5.1,3.4 md
	2.2 md	2.4,2.1 md
	1.3 md	1.5,1.4 md
Total MW	54.5 md	54.5 md
		48 md

Attempts by several individuals on different occasions to detect the transfer of plasmid DNA into recipient organisms has met with only partial success. In early trials we relied upon proven donor and recipient strains which were received from Dr. J. M. Pemberton. The donor strain would transfer pJMP397 into Alcaligenes strain JMP228. The pJMP397 carries known markers specifying resistance to 40 µg/ml of $HgCl_2$ and confers the ability to utilize 2,4-D. Transfer of this plasmid was successfully completed in this laboratory. However, attempts to transfer 2,4-D degrading ability from most cultures isolated in Oregon into culture 228 did not meet with success. Cultures EWC, ERC, EML155, and EML157 served as potential donors in these experiments, while Alcaligenes JMP228 served as the recipient. Crosses were made using a variety of incubation conditions including prolonged overnight exposures of mating partners in broth and on agar surfaces. Potential transconjugants were screened for resistance to mercury or 2,4-D utilization. Neither were detected.

It is significant that transconjugants were obtained in the transfer of pEML159 from the Alcaligenes EML159 strain isolated from the Corvallis wastewater plant. Transfer was accomplished into Alcaligenes JMP228 which acquired both the resistance to mercury as well as the 2,4-D utilizing ability. This is an important observation because of the similarities in restriction fragment sizes, the % G+C, and known phenotypic markers which are on both plasmids which exhibited transfer capabilities, i.e., pJMP397 and pEML159. It is also most interesting that two plasmids derived from bacteria isolated on different continents would have

so many molecular similarities. Efforts are ongoing to identify additional transmissable plasmids of the pEML159 type among other isolates which degrade 2,4-D.

We also tested the possible transmission of plasmids conferring 2,4-D utilization into other commonly occurring gram-negative rods isolated from aquatic environments. So far, we have not been able to transfer pEML159 into any of these bacteria.

Six cultures were tested for their metabolic activity for utilizing pesticides other than 2,4-D (Table 5). Compounds tested included various mono- and dichloro-substituted phenols as well as two common pesticides, Sevin and Amitrol-T. Little or no activity was detected on nearly all these compounds, when present at 150 µg/ml of medium. However, when the pesticide concentration was reduced to 75 µg/ml, some were utilized. In addition to 2,4-D, 2,4-dichlorophenol and 4-chlorophenol were utilized most rapidly and most completely by nearly all the isolates. Two-chlorophenol, 3,4- and 2,6-dichlorophenol were not utilized. The 2,3- and 2,5-dichlorophenol were probably modified by most cultures since there was a shift in peak absorption to shorter wavelengths although there was no significant decrease in peak heights.

It is not surprising that 2,4-dichlorophenol is used since it is probably the first intermediate breakdown product of 2,4-D (21,22). The 2,4-dichlorophenol breakdown product is 4-

Table 5. Patterns of utilization of 2,4-D and other pesticides by selected pure cultures.

Pesticide	Culture EWC	EWD	EMA	ERA	ERB	ERC
2,4-dichloro-phenoxyacetic acid	++	++	++	++	++	++
2,4-dichlorophenol	+	+	+	+	+	+
2-chlorophenol	-	-	-	-	-	-
4-chlorophenol	++	++	++	++	±	+
2,3-dichlorophenol	±	±	±	±	-	±
2,5-dichlorophenol	±	±	±	±	±	±

[a] ++ designates complete utilization of the compound, + designates 25-75% reduction in peak height from u.v. scans, ± designates less than 25% reduction in peak, usually accompanied by a shift in wavelength of peak absorption. Cultures were incubated up to 7 weeks in some trials. Additional compounds tested which were not utilized include: 2,6-dichlorophenol, 3,4-dichlorophenol, penta chlorophenol, carbaryl (Sevin), and 3-amino-1,2,4-trizole (Amitrol-T).

chlorocatechol. The latter is the first intermediate of the hydroxylase activity on 4-chlorophenol which was also rapidly utilized (23). The partial degradation or lack of action on the 2-, and 2,3-chlorophenols probably reflects steric hindrance on the enzyme which dechlorinates the 2,4- and 4-chloroisomers.

REFERENCES

1. Dunnette, D. A. 1978. Overview: Our toxicological dilemma. In: Toxic materials in the aquatic environment. Water Resources Research Institute, Oregon State University, Corvallis, OR
2. Environmental quality. 1979. The tenth annual report on the council on environmental quality. 816 pp. U.S. Government Printing Office No. 041-011-0047-5.
3. Sommerville, H. J. 1978. Pesticides, microorganisms, and the environment. Span. 21:35-37.
4. Alexander, M. 1981. Biodegradation of chemicals of environmental concern. Science 211:132-134.
5. Alexander, M. 1979. Helpful, harmful, and fallible microorganisms: importance in transformation of chemical pollutants. In: Aquatic Microbial Ecology, Proc. American Society for Microbiology Conference, Clearwater Beach, Fl. Univ. Maryland Sea Grant Publ.
6. Tiedje, J. M. 1979. An attempt at identifying research needs for studies on microbial transformations. In: Aquatic Microbial Ecology, Proc. American Society for Microbiology Conference, Clearwater Beach, Fl. Maryland Sea Grant Publ.
7. Chatterjee, D. K., S. T. Kellogg, S. Hamada,and A. M. Chakrabarty. 1981. Plasmid specifying total degradation of 3-chlorobenzoate by a modified ortho pathway. J. Bacteriol. 146:639-646.

8. Fisher, P. R., J. Appleton, and J. M. Pemberton. 1978. Isolation and characterization of the pesticide-degrading plasmid pJP1 from Alcaligenes paradoxus. J. Bacteriol. 135:798-804.

9. Don, R. H., and J. M. Pemberton. 1981. Properties of 6 pesticide degradation plasmids isolated from Alcaligenes paradoxus and Alcaligenes eutrophus. J. Bacteriol. 145:681-686.

10. Kellog, S. T., D. K. Chatterjee, andA. M. Chakrabarty. 1981. Plasmid-assisted molecular breeding: new technique for enhanced biodegradation of persistent toxic chemicals. Science 214:1133-1135.

11. Kemp, P. F., and A. M. Chakrabarty. 1979. Plasmids specifying p-chlorobiphenyl degradation in bacteria. p. 275-285. In: K. N. Timmis and A. Puhlerled (ed.). Plasmids of medical, environmental and commercial importance. Elsevier/North-Holland Biomedical Press, Amsterdam.

12. Aaronson, S. 1970. Experimental Microbial Ecology. pp. 91-92. Academic Press, New York, New York.

13. MacFaddin, J. F. 1977. Biochemical tests for identification of medical bacteria. William and Wilkinson Co., Baltimore, MD.

14. Johnson, J. L. 1981. Genetic characterization, p. 450-472. In P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.). Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.

15. Mandel, M. L., L. Igambi, J. Bergendahl, M. L. Dodson, Jr., and E. Scheltgen. 1970. Correlation of melting temperature and cesium chloride buoyant density of bacterial deoxyribonucleic acid. J. Bacteriol. 101:333-338.

16. Hinshaw, V., J. Punch, M. J. Allison,a nd H. P. Dalton. 1969. Frequency of R-factor-mediated multiple drug resistance in Klebsiella and Aerobacter. Appl. Microbiol. 17:214-218.

17. Talbot, H. W., D. K. Yamamoto, M. W. Smith, and R. J. Seidler. 1980. Antibiotic resistance and its transfer among clinical and nonclinical Klebsiella strains in botanical environments. Appl. Environ. Microbiol. 39:97-104.

18. Coplin, D. L. 1978. Properties of F and P group plasmids in Erwinia stewartii. Phytopathol. 68:1637-1643.

19. Jorgensen, S. T., and A. L. Poulsen. 1976. Antibiotic resistance and Hly plasmids in serotypes of Escherichia coli associated with procine enteric disease. Antimicrob. Agents Chemother. 9:6-10.

20. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning. Cold Spg. Harbor Lab.

21. Tiedje, J. M., J. M. Duxbury, M. Alexander, and J. E. Dawson. 1969. 2,4-D metabolism: Pathway of degradation of chlorocatechols by Arthrobacter sp. J. Agr. Food Chem. 17:1021-1026.

22. Tiedje, J. M., and M. Alexander. 1969. Enzymatic cleavage of the ether bond of 2,4-dichlorophenoxyacetate. J. Agr. Food Chem. 17:1080-1085.

23. Steenson, T. I., and N. Walker. 1957. The pathway of breakdown of 2,4-dichloro- and 4-chloro-2 methylphenoxyacetic acid by bacteria. J. Gen. Microbiol. 16:146.