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Characterization of Aquatic Bacteria and Cloning of Genes Specifying Partial Degradation of 2,4-Dichlorophenoxyacetic Acid†

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Water samples from rivers, streams, ponds, and activated sewage were tested for the presence of bacteria which utilize 2,4-dichlorophenoxyacetic acid (2,4-D) as a sole source of carbon. Seventy percent of the attempted enrichments yielded pure cultures of 2,4-D-metabolizing bacteria. All but 1 of the 30 isolates were gram-negative rods, all but 2 were motile, and all were nonfermentative and oxidase and catalase positive. Nine isolates had DNA guanine-plus-cytosine values of 61.1 to 65 mol%. One isolate had a 67 mol% guanine-plus-cytosine value. The results suggest that these 2,4-D-metabolizing bacteria belong to the genus *Alcaligenes*. Fourteen of 23 isolates contained one or more detectable plasmids of about 20, 60, or 100 megadaltons. *Hin*dIII restriction fragment patterns showed these plasmids to be different from each other with one exception. Very similar restriction fragment patterns were revealed with a plasmid isolated from an *Alcaligenes eutrophus* strain obtained from Australia (pJMP397) and in an *Alcaligenes* sp. isolated in Oregon (pEML159). These two plasmids were about 56 megadaltons, had the same guanine-plus-cytosine value, were transmissable, and coded for 2,4-D metabolism and resistance to $HgCl_2$. Hybridization of these two plasmids was demonstrated by using nick-translated ^{32}P-labeled pJMP397. The vector pBR325 was used to clone *Hin*dIII fragments from pEML159. One cloned fragment of 14.8 megadaltons expressed in *Escherichia coli* the ability to release $^{14}CO_2$ from 2,4-D labeled in the acetate portion.

Synthetic chemicals are a beneficial and necessary part of life in any modern society. Over 800,000,000 lb (362,873,600 kg) of pesticide products enter our environment annually (1). Some 37 to 56 million tons (ca. 336×10^6 to 508×10^6 t) of waste generated annually in this country is considered hazardous (9). Biological toxicity is only one important aspect in understanding the environmental impact of toxic xenobiotics. To further understand the potential environmental concerns, we also need to know their fate in the environment. Degradation of toxic substances may result from biotic or abiotic activities. The latter include photooxidation and adsorption to particulates (27). However, the abiotic processes only contribute to partial degradation (never to mineralization). Microbial action on xenobiotics is probably the most important consideration in their elimination from soil and aquatic ecosystems.

Scientists have argued that there is a critical need in research to develop new synthetic pesticide compounds which are biodegradable (30). One such compound already widely used is 2,4-dichlorophenoxyacetic acid (2,4-D). Bacterial genera such as *Pseudomonas*, *Acinetobacter*, *Arthrobacter*, *Corynebacterium*, and *Alcaligenes* have been shown to metabolize the herbicide 2,4-D (3, 4, 11–14, 28, 31–33). Strains which metabolize chlorinated phenols may possess plasmids encoding this capability (11, 24). Australian researchers have isolated *Alcaligenes* sp. which contain a plasmid coding for 2,4-D metabolism (11, 14). They have found that plasmids of the IncP1 grouping were transferred to other *Alcaligenes* species and to other genera. These plasmids were for the most part cryptic except for resistance to $HgCl_2$ and metabolism of 2,4-D and related compounds (11).

Little is known about the relationships between the rate of xenobiotic degradation in the environment and the type, numbers, and functions of bacteria involved in the process. In the present study the occurrence of bacteria which metabolize 2,4-D as a sole source of carbon were isolated from a variety of sources and phenotypically and genetically characterized. The gene(s) which codes for the removal of the acetate residue from 2,4-D was cloned into *Escherichia coli* from a 56-megadalton (Mdal) plasmid present in one of the isolates. The cloned gene(s) will prove useful in evaluating the genetic diversity of elements in other bacteria which metabolize 2,4-D.

MATERIALS AND METHODS

Media and solutions. Enrichment medium (EM) was used throughout this study (1). The contents are as follows: K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; $FeCl_2$, 0.02 g, NH_4SO_4, 1 g; and distilled water to 1 liter. EM without an added carbon source was used as a wash for cell suspensions in the ^{14}C-labeled 2,4-D assay. EM with an added carbon source was used for initial bacterial enrichments, general bacterial growth, and testing other chlorinated hydrocarbons for degradation. Noble agar (1.2%) was added to solidify the medium. Urea slants, oxidative-fermentative tubes, Simms motility test medium, and Simmons citrate agar slants were prepared as recommended by the supplier (Difco Laboratories). Luria-Bertoli broth (LB) was used for general bacterial growth and as the base for antibiotic-containing agar plates. Each xenobiotic was prepared as a 20-mg/ml stock in 0.1 M NaH_2PO_4, the pH was adjusted to pH 7.0 for maximum solubility, and the stock was filter sterilized before use.

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Glutaraldehyde, electron microscopy grade, was purchased from Polysciences, Inc. Tris buffers used in plasmid extractions contained 0.05 M Tris–0.01 M trisodium EDTA (TE) and 0.05 M Tris–0.005 M trisodium EDTA–0.05 M NaCl (TES); both were adjusted to pH 8.0. Dilutions of standard saline citrate buffer (SSC; 20× SSC contains 175.3 g of NaCl plus 88.2 g of sodium citrate, in 1 liter of distilled water, pH 7.0) were used in DNA blotting and hybridization and for DNA thermal denaturation experiments. Selected antibiotics were prepared as follows: ampicillin, 25 mg/ml in 0.1 M potassium phosphate buffer at pH 7.0; chloramphenicol, 34 mg/ml in 100% ethanol; tetracycline, 10 mg/ml in 100% ethanol; nalidixic acid, 20 mg/ml in water. Transformation buffer containing 0.075 M $CaCl_2$, 0.01 M morpholinepropanesulfonic acid (Sigma Chemical Co.), and 0.5% glucose, pH 6.5, was used for DNA transformation procedures and in the ^{14}C-labeled 2,4-D assay to make strains of *E. coli* permeable to 2,4-D. Electrophoresis buffer (TAE) was prepared as described by Maniatis et al. (23).

Bacterial cultures. Cultures designated JMP134, a 2,4-D-degrading *Alcaligenes* sp.; JMP228, a plasmid-cured *Alcaligenes* sp.; and JMP397, an *E. coli* containing pJP4 carrying genetic information for the degradation of 2,4-D, were provided by J. M. Pemberton. Sometime after its arrival in this laboratory, we noted that JMP397 contained a second plasmid of unknown origin and function. When whole-plasmid extracts are described in this report, they are referred to as pJMP397; when the specific 2,4-D-degrading plasmid is described, it is referred to as pJP4, using the terminology established by Don and Pemberton (11). *Pseudomonas syringe* pv. *phaseolicola* LR700 was provided by Dallice Mills, Oregon State University. PSA122 is a strain of *E. coli* HB101 containing a hybrid plasmid prepared in this study, using an *Alcaligenes* sp. plasmid restriction fragment and vector plasmid pBR325.

Bacterial enrichments. Thirty bacterial cultures, capable of utilizing 2,4-D as their sole carbon source, were isolated from western Oregon natural waters and returned activated sludge. Each 100-ml water sample was placed in a 250-ml Erlenmeyer flask to which was added 150 μg of 2,4-D per ml. The cultures were shaken at 200 rpm and 30°C for 1 week, after which the degradation of 2,4-D was determined spectrophotometrically at 283.35 nm, using a Beckman DU 8 programmable spectrophotometer. One milliliter of this enrichment culture was centrifuged (Beckman microcentrifuge B) for 5 min to sediment the cellular material and any debris. A sample of the supernatant was used to measure the absorbance peak of 2,4-D at 283.35 nm. If a decrease in absorbance at the specific wavelength had occurred, 0.5 ml was transferred to a defined enrichment medium (100 ml of EM) containing 150 μg of 2,4-D per ml. This was incubated as above for 1 week. After the second loss of absorbance, the broth was streaked to EM Noble agar plates (EM agar) containing 150 μg of 2,4-D per ml. The plates were incubated at 30°C until colonies were large enough to be picked and restreaked. Cultures were then grown in either EM broth with up to 400 μg of 2,4-D per ml or LB until they reached late log phase, diluted 1:1 with 20% sterile glycerol, and stored at −80°C.

Characterization of cultures. Each culture was tested by Gram stain, oxidase test (1% tetramethyl *p*-phenylenediamine; Sigma), catalase (3% hydrogen peroxide), growth on Simmons citrate agar slants, growth at selected temperatures, and presence of pigment on LB agar plates, Simms motility test medium, and oxidative-fermentative tubes containing glucose as a fermentable substrate. Selected isolates were screened by API 20E test strips (Diagnostic Strips, Analylab Products, Inc.), urea slants, and LB agar containing filter-sterilized antibiotics and other inhibitory compounds such as arsenite and $HgCl_2$ (40 μg/ml).

Electron microscopy was done to determine flagellar arrangement of selected isolates. Cells were grown in 1:5 distilled water-diluted LB broth without NaCl to mid-log phase in petri dishes at 30°C, fixed with 1% glutaraldehyde, gently transferred to 30-ml Corex glass centrifuge tubes, and centrifuged at 1,940 × *g* for 5 min. The supernatant was decanted, and the cells were overlaid with sterile distilled water and allowed to suspend overnight at 4°C. Drops of cell suspension were placed on Formvar-coated copper grids and shadow coated with chromium in a Varian VE 10 vacuum evaporator. The cells were photographed with a Phillips EM300 microscope.

Chromosomal DNA from selected isolates was purified by the method of Seidler and Mandel (26). Chromosome and plasmid guanine-plus-cytosine content (G+C) compared with *E. coli* WP2 was determined by the thermal melting technique, using a Beckman DU 8 programmable spectrophotometer (22).

Plasmid DNA. Plasmid DNA was extracted in small quantities by modifying the method of Portnoy and White (10). The modifications were made to accommodate the difficult-to-lyse 2,4-D-degrading isolates. The pH of the lysis buffer was adjusted to 12.7 for 2,4-D-degrading isolates and 12.4 for *E. coli* cultures (measured by a Fisher Scientific Tris buffer pH electrode). After 4 h on ice, microfuge tubes were placed in adapters for the Beckman rotor JA-20 and centrifuged for 10 min at 7,700 × *g*. The supernatant was removed and its volume was determined. Then 0.55 volume of isopropanol (−20°C) was added to the mixture, placed at −80°C for 5 min, and microfuged for 5 min, and the pellet was washed once with 100% cold ethanol (−20°C) before vacuum drying. The pellet was allowed to resuspend overnight in a volume of 20 to 50 μl of water or buffer of choice.

Large-scale extractions of plasmid DNA were done with 100 ml of culture, using the same method, but included an RNase treatment (40 μl of a 10-mg/ml stock solution RNase [Sigma] at 40°C for 20 min) performed after the isopropanol precipitation and suspension of the pellet in 4 ml of TES. To each 4 ml of RNase-treated DNA, 4.2 g of CsCl (Bethesda Research Laboratories) was added. The mixture was placed in a Beckman Quik Seal ultracentrifuge tube and submitted to ultracentrifugation at 65,000 rpm for 15 h at 15°C in a Beckman VTi 80 rotor. DNA bands which formed during centrifugation were removed, the ethidium bromide was extracted with TES-saturated isoamyl alcohol, and the DNA was dialyzed against TE buffer. If found to be contaminated with chromosomal DNA, samples were rebanded by ultracentrifugation.

DNA samples were visualized by gel electrophoresis and photography, using a Polaroid camera and a UV transilluminator (Ultra Violet Products, Inc.). A horizontal electrophoresis unit (Bethesda Research Laboratories) and agarose concentrations of 0.6 to 0.8% were used to screen plasmid preparations and to separate restriction fragments.

Restriction enzyme analyses were done as recommended by the manufacturer (Bethesda Research Laboratories). At completion, 0.2 volume of stop mix (0.07% bromphenol blue, 7% sodium dodecyl sulfate, 20% Ficoll) was added before separation on agarose gels.

DNA dot blots were prepared as described by Kafatos et al. (17), except that DNA samples were denatured with 0.1 volume of 4 N NaOH for 10 min and neutralized by 0.1

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

Isolate	Source
EML130	Activated sludge (static), Corvallis, Ore.
EML131	Activated sludge (shaker), Corvallis, Ore.
EML132	Mary's River (static), Corvallis, Ore.
EML133	Mary's River (shaker), Corvallis, Ore.
EML134	Willamette River (static), Corvallis, Ore.
EML135	Willamette River (shaker), Corvallis, Ore.
EML137	Pond (shaker)
EML140	Mary's River (static), Philomath, Ore.
EML141	Mary's River (shaker), Philomath, Ore.
EML142	Pond (static), Philomath, Ore.
EML143	Pond (shaker), Philomath, Ore.
EML144	Pond (static), Corvallis, Ore.
EML145	Pond (shaker), Corvallis, Ore.
EML146	Willamette River, Corvallis, Ore.
EML147	Standing water near Willamette, Corvallis, Ore.
EML148	Jaunt Creek (sediment), Polk County, Ore.
EML149	Jaunt Creek (running water), Polk County, Ore.
EML155	Standing water, Gresham, Ore.
EML157	Return activated sludge, Corvallis, Ore.
EML158	Willamette River, Corvallis, Ore.
EML159	Return activated sludge, Corvallis, Ore.
EML160	Willamette River, Corvallis, Ore.
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

volume of 4 M ammonium acetate. Prehybridization, hybridization, and nick translation procedures of Maniatis et al. (23) were used.

Bacterial conjugation and transformation. Plasmids from JMP397, an *E. coli* strain containing pJP4, and EML159 were transferred to a cured strain of *Alcaligenes* sp., JMP228, by growing the desired cultures to mid-log phase with shaking at 35°C in LB, mixing the cultures so that donors outnumbered recipients by 10:1, and placing this mixture in a flask so that no more than 10 ml covered the bottom of a 150-ml Erlenmeyer flask. These conjugation mixtures were incubated statically at 35°C for up to 20 h. Samples were then spread plated on LB agar or EM Noble agar containing antibiotics (15) or $HgCl_2$ (40 μg/ml).

Transformation of plasmid DNA from JMP397 into *E. coli* HB101 and transformation of HB101 by pPSA122 were performed by the method of Cohen et al. (7).

Cloning of genes expressing 2,4-D degradative activity was accomplished by digesting pEML159 and vector pBR325 with the restriction endonuclease *Hin*dIII. After ligation of the fragments (23), DNA was transformed into HB101 as described above. After expression of the plasmid in its new host, cells were plated on LB agar containing 25 μg of chloramphenicol and 200 μg of ampicillin per ml. Colonies unable to grow on LB agar plates containing 15 μg of tetracycline per ml were prepared for small-scale plasmid extraction and examined for evidence of cloned DNA in vector pBR325.

Radioisotopic 2,4-D assay. Cultures containing a hybrid plasmid were tested for ability to release $^{14}CO_2$ from ^{14}C-labeled 2,4-D. Cells grown in LB broth overnight were washed once in EM and then suspended in a mixture of 50% EM and 50% transformation buffer. A 10-ml portion was placed in 50-ml serum vials; 0.1 μCi of ^{14}C-labeled 2,4-D (2,4-dichlorophenoxy [2-^{14}C]acetic acid; specific activity, 55 mCi/mmol; Amersham Corp.) and 10 μg of unlabeled 2,4-D per ml were added. Each vial was fitted with a serum stopper with a bucket attachment (Kontes) containing a fluted Whatman no. 1 paper. The serum vials were gently shaken on a table-top shaker (Labline, Junior Orbiter) for 20 h. The reaction was then stopped by injection of 0.5 ml of 2 N H_2SO_4. Beta-phenylethylamine was injected to just wet the fluted filter paper (0.15 ml), and the serum vials were returned to the shaker for 1 h. The fluted filter papers were removed, placed in 5 ml of Omnifluor, and counted for radioactivity trapped as $^{14}CO_2$ in the filter paper (2).

Utilization of other chlorinated hydrocarbons. Selected isolates were grown in 5 ml of LB broth overnight, washed twice in EM without 2,4-D (Beckman J2-21; 3,000 × *g*), and suspended in 5 ml of EM without 2,4-D. Samples (0.5 ml) of the cell suspension were placed in sterile screw-cap tubes containing 5 ml of EM and the appropriate chlorinated hydrocarbon at 50 μg/ml. These cultures were gently shaken for 2 weeks at 30°C, and then absorbance of the culture fluid was measured at appropriate wavelengths with a Beckman DU 8 spectrophotometer. The suspensions were also tested for 2,4-D utilization as described above. Control flasks contained uninoculated EM and the appropriate chlorinated hydrocarbon.

RESULTS

Thirty bacterial isolates which utilize 2,4-D as a sole source of carbon and energy were obtained from 45 enrichment cultures. Specimens from returned activated sewage were consistently positive, as were water samples collected in agricultural areas (Table 1). All isolates were capable of growth at the expense of 2,4-D (150 μg/ml) as evidenced by the total loss of UV-absorbing compounds (250 to 290 nm) and by an increase in biomass produced during passages through EM containing only 2,4-D as a carbon source.

When low levels (1 to 10 μg/ml) of 2,4-D were used in enrichment cultures, 10- to 100-fold-higher most-probable-number values were demonstrated for the disappearance of 2,4-D. Although these results imply a toxicity of 2,4-D at higher concentrations (150 μg/ml), when samples were plated on 2,4-D Noble agar from enrichments containing 1 to 10 μg of 2,4-D per ml, colonies of 2,4-D-metabolizing bacteria were not detected.

The 30 isolates were sorted into two groups based on the presence or absence of carotenoid pigment production on either LB or EM Noble agar. Further differences were observed when all 30 cultures were tested for citrate utilization and maximum temperature of growth. Figure 1 is a flow chart of the phenotypic differentiation of environmental isolates which degrade 2,4-D.

All isolates were oxidase and catalase positive and oxidation-fermentation negative. API 20E strips further demonstrated the nonfermentative nature of our isolates and added very little to the overall phenotypic profiles. Traits shown in parentheses in Fig. 1 were demonstrated by the given isolate from API results and represent uncommon occurrences. Twenty-nine isolates were gram-negative rods, occurring as single or short chains. The one gram-positive isolate lost its

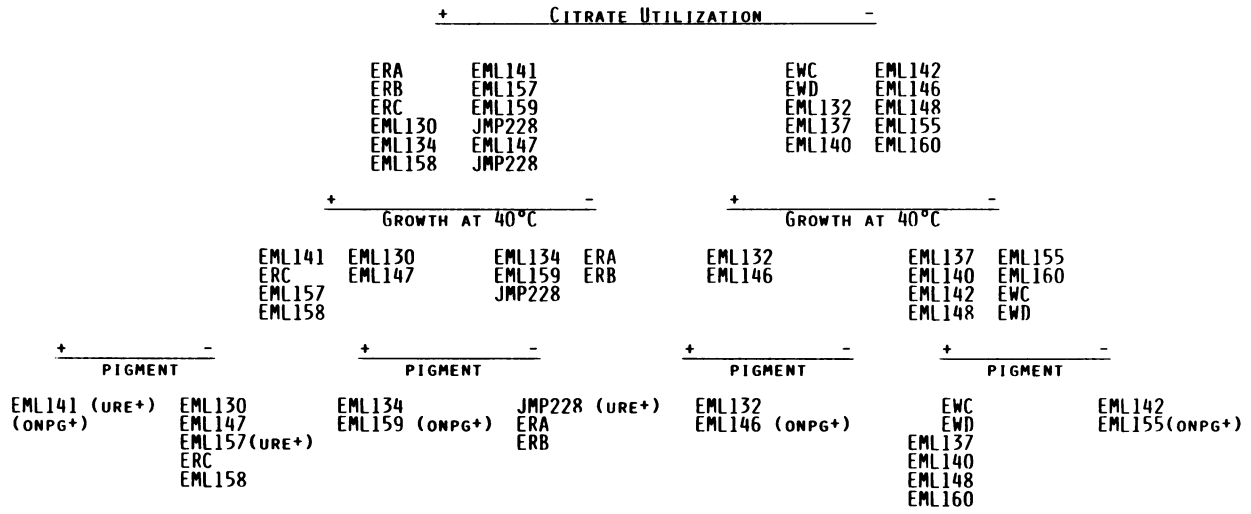

FIG. 1. Flowchart of phenotypic traits of environmental isolates and JMP cultures. Isolates were grouped according to citrate utilization, growth at 40°C, and production of pigment. Tests in parentheses were determined with API strips and represented unique features of individual strains.

ability to degrade 2,4-D after only a few transfers and was not further characterized. All but two isolates were motile by observation under oil immersion and with Simms motility test medium. Electron microscopic observations revealed that the most common site of flagellar attachment was subpolar with an occasional lateral flagellum on some cells.

Chromosomal DNA G+C was in a range of 61.1 to 66.9 mol% for selected isolates from this study and previously described *Alcaligenes* sp. which utilize 2,4-D (Table 2). The average G+C of all isolates excluding strains ERB and EML158 is 62.6 mol%. These two strains have significantly higher G+C contents of 66.9 and 64.6 mol%, respectively.

In an attempt to gather insight into the most common 2,4-D-degrading pathway used by these isolates, several other chlorinated hydrocarbons with structures similar to 2,4-D were tested for degradation. Patterns of chlorinated hydrocarbon utilization are shown in Table 3. Attempts to grow isolates on 2,4-dichlorophenol (2,4-DCP) at 150 μg/ml failed due to the toxicity of this compound compared with 2,4-D. When the concentration was lowered to 50 μg/ml, all tested isolates could metabolize 2,4-DCP and most used 4-chlorophenol. 2-Chlorophenol was not used and the isolates did not completely metabolize other isomers of the chlorinated phenols.

Twenty-three isolates were tested for the presence of plasmids. Fourteen (61%) were shown to contain one or more detectable plasmid by our extraction procedure. In Fig. 2, selected plasmids are shown after separation by gel electrophoresis. The plasmids range from 5 to 112 Mdal, with sizes clustering around 20, 60, and 100 Mdal. Many isolates contain multiple plasmids which band together during ultracentrifugation in CsCl. Although antibiotic resistance patterns of the isolates vary greatly, approximately 50% of the isolates are resistant to 40 μg of $HgCl_2$ per ml. However, most of the plasmids remain cryptic since all attempts at curing selected isolates by using mitomycin C (14) have failed.

*Hin*dIII restriction endonuclease fragment patterns clearly show that most of these plasmids are different from each other (Fig. 3). Similar endonuclease fragment patterns are seen with pEML159 and pJMP397. Plasmids from these two bacterial strains are compared further in Fig. 4. JMP397 contains two plasmids of very similar size so that upon restriction endonuclease digestion more fragments can be seen with this strain than are seen in isolate EML159. A second restriction endonuclease digestion, *Eco*RI, again yielded several fragments of identical molecular weight. By whole-plasmid size determination both JMP397 and EML159

TABLE 2. G+C content of chromosomal DNA from selected 2,4-D-metabolizing isolates

Isolate	G+C (mol %; ± SD)[a]
JMP 228	62.7 ± 0.3
JMP 134	63.7 ± 0.7
EWC	61.6 ± 0.4
ERC	63.7 ± 0.7
ERB	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

[a] Values are means of three determinations.

TABLE 3. Patterns of utilization of 2,4-D and other pesticides by selected pure cultures

Pesticide	Utilization by given culture[a]					
	EWD	EMA	ERA	ERB	ERC	EML159
2,4-D	++	++	++	++	++	++++
2,4-DCP	+	+	+	+	+	++
2-Chlorophenol	−	−	−	−	−	−−
4-Chlorophenol	++	++	++	++	±	+±
2,3-Dichlorophenol	±	±	±	±	−	±±
2,5-Dichlorophenol	±	±	±	±	±	±±

[a] ++, Complete utilizaton of the compound; +, 25 to 75% reduction in peak height from UV scans; ±, <25% reduction in peak, usually accompanied by a shift in wavelength of peak absorption. Cultures were incubated for up to 7 weeks in some trials. Additional compounds tested which were not utilized include 2,6-dichlorophenol, 3,4-dichlorophenol, and pentachlorophenol.

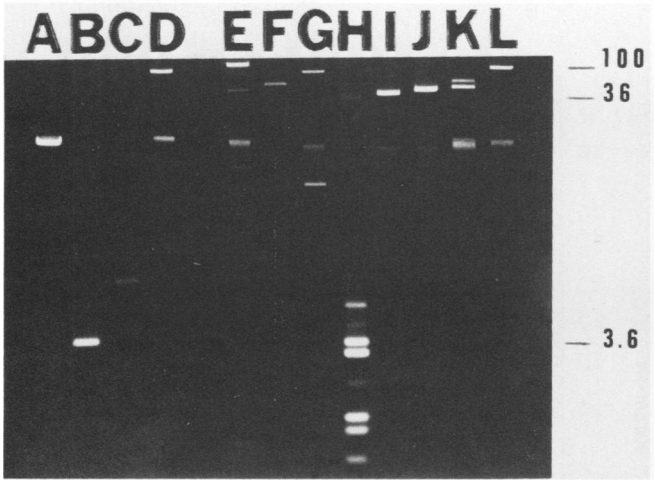

FIG. 2. Plasmids from selected isolates and molecular weight markers after separation by agarose gel electrophoresis. Values are expressed in megadaltons. Lanes: (A) lambda DNA, 33; (B) pBR325, 3.6; (C) pERC, 5.5; (D) pMC7105, 99.7; (E) pEML141, 112, 83, 56, 17; (F) pEML146, 60, 21; (G) pEML148, 96, 13; (H) *E. coli* V517 top band, 35.8; (I) pEML155, 50; (J) pEML159, 56; (K) pJMP397, 66, 56; (L) pMC7105, 99.7. Other undesignated bands in lanes D, E, K, and L represent chromosomal DNA. *Pseudomonas* sp. strain LR700(pMC7105), pBR325, undigested phage lambda DNA, and *E. coli* strain V517 (21) were used as molecular weight markers. A graph of the log molecular weight versus log millimeters of migration from the origin was prepared to determine the molecular weight of the unknown plasmids.

contain a plasmid of 56 Mdal, and JMP397 contains an additional plasmid of 66 Mdal.

Further evidence of similarity between pEML159 and pJMP397 is demonstrated by the DNA thermal denaturation

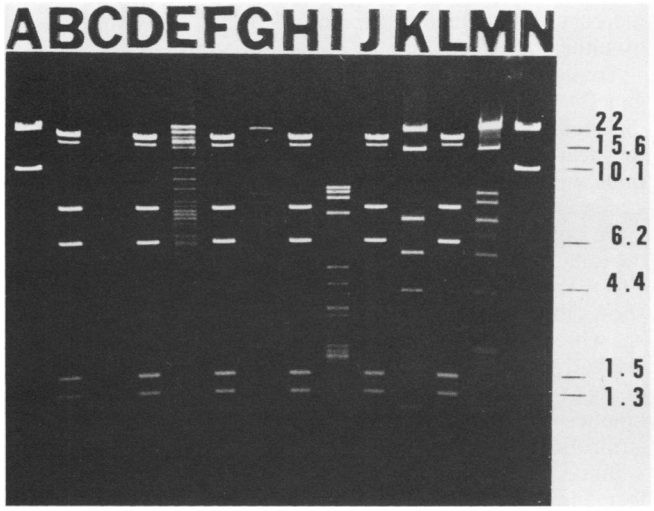

FIG. 3. *Hin*dIII restriction analysis of plasmids from selected isolates from each phenotypic group. Lanes: (A and N) lambda DNA, *Sal*I digestion; (B, D, F, H, J, L) lambda DNA, *Hin*dIII digestion; (C) pERC; (E) pEML141; (G) pEML146; (I) pEML155; (K) pEML159; (M) pJMP397. Lambda DNA was used as molecular weight markers and the dominant band sizes are listed on the right; the values are in megadaltons. The *Hin*dIII fragment of lambda DNA above the 15.6-Mdal fragment is due to incomplete digestion of the molecule. It becomes evident only in high-resolution, low-voltage electrophoresis.

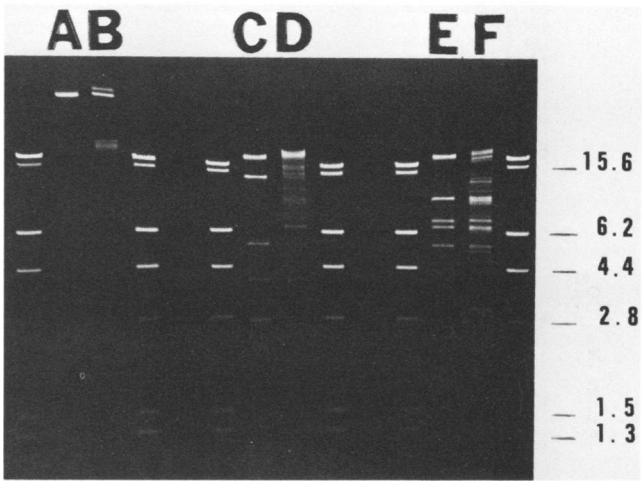

FIG. 4. Comparison of pJMP397 and pEML159 by size of undigested plasmid and restriction patterns. Intact plasmids are shown in lanes A (pEML159) and B (pJMP397). *Hin*dIII digestions are shown in lanes C (pEML159) and D (pJMP397). *Eco*RI digestions are shown in lanes E (pEML159) and F (pJMP397). *Hin*dIII-digested lambda DNA was used for molecular weight standards and the sizes are given in megadaltons.

curves for these two plasmids. The T_m was identical for both plasmid preparations (79°C) and corresponds to 57 mol% G+C. In addition, hybridization of the two plasmid DNAs revealed significant reassociation as illustrated in the dot blot shown in Fig. 5. Purified plasmid preparations from three other 2,4-D-metabolizing isolates failed to hybridize with the DNA probe prepared from pJMP397.

The transfer of pJMP397 or pEML159 into the cured *Alcaligenes* sp. strain JMP228 have further demonstrated the similarity of these two plasmids. The transfer of either plasmid conferred the ability to degrade 2,4-D and resistance to $HgCl_2$. However, subtle differences do exist since

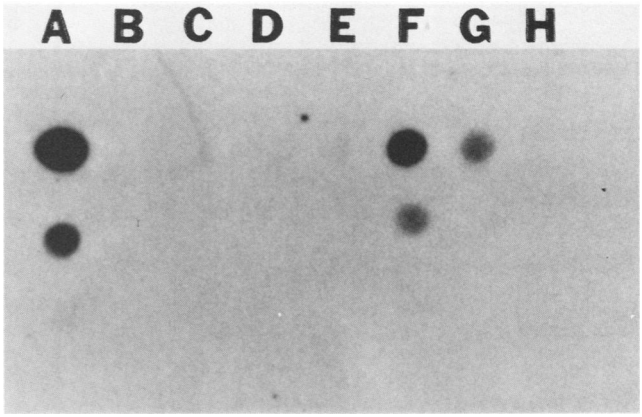

FIG. 5. Dot blot hybridization, using nick-translated ^{32}P-labeled pJMP397 as the DNA probe. The nitrocellulose filter was incubated for 20 h at 42°C with 30% formamide. DNA concentrations in purified samples were determined by the optical density at 260 (OD_{260}) nm and converted to micrograms per milliliter by the formula 50 μg of DNA per ml = 1.0 OD_{260}. Lanes: (A) pJMP397; (B) calf thymus; (C) pERC; (D) pEWC; (E) pEML155; (F) pEML159; (G) JMP228; (H) water blank. A 0.1-μg amount of unlabeled DNA was spotted in the upper row and 0.01 μg was spotted in the lower row.

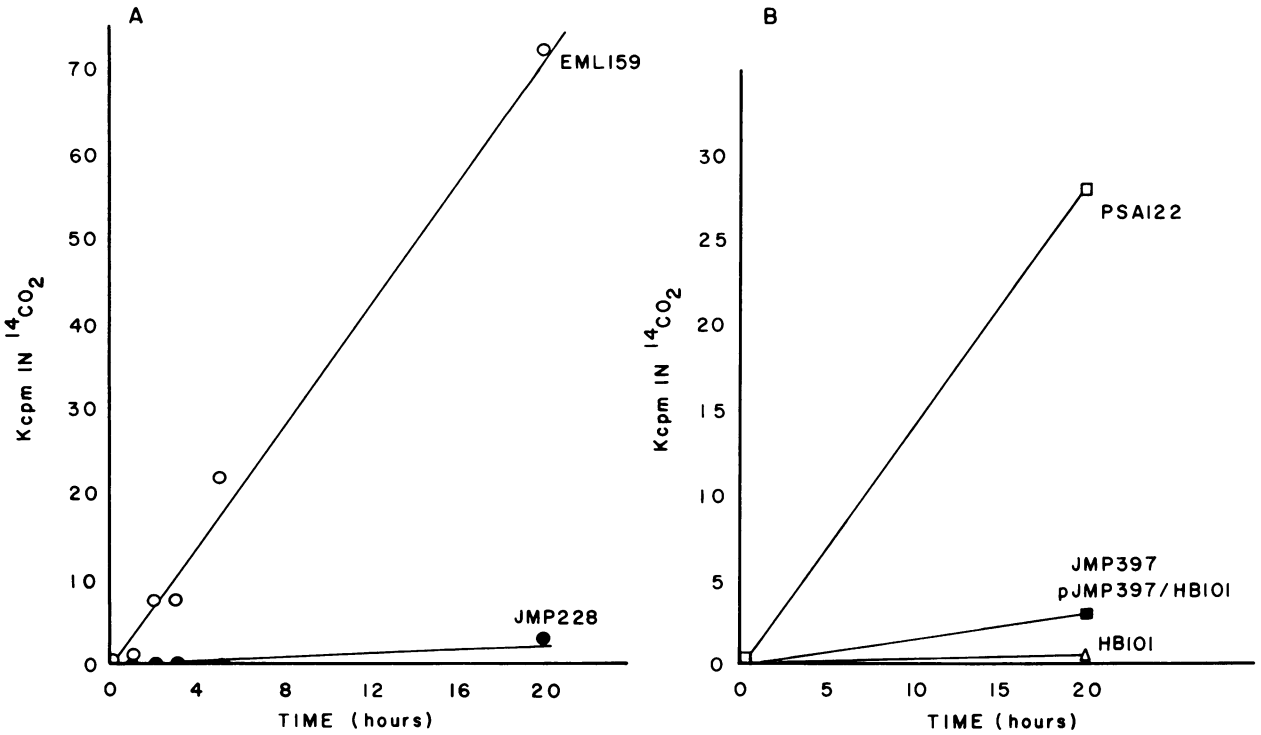

FIG. 6. Release of $^{14}CO_2$ from ^{14}C-labeled 2,4-D. Selected strains were grown in LB overnight, washed in EM, and suspended to the desired cell density (optical density at 600 nm [OD_{600}]) in EM plus 10 μg of unlabeled 2,4-D per ml. To this was added 0.1 μCi of ^{14}C-labeled 2,4-D in a 7.5-ml total volume. Samples were prepared in duplicate for each time point. Zero time average values were subtracted from incubated samples. (A) Linear release of $^{14}CO_2$ was seen in a time course experiment with the positive control *Alcaligenes* sp. strain EML159 (○); negative control was *Alcaligenes* sp. strain JMP228 (●). Cell densities were OD_{600} = 0.06 to 0.08. (B) *E. coli* strains with or without plasmids were compared for ability to release $^{14}CO_2$ from ^{14}C-labeled 2,4-D. Cell densities were OD_{600} = 0.62 to 0.8. With strains of *E. coli*, cells were made permeable with 1:1 (vol/vol) cell suspension in EM to transformation buffer ($CaCl_2$-morpholinepropanesulfonic acid-glucose).

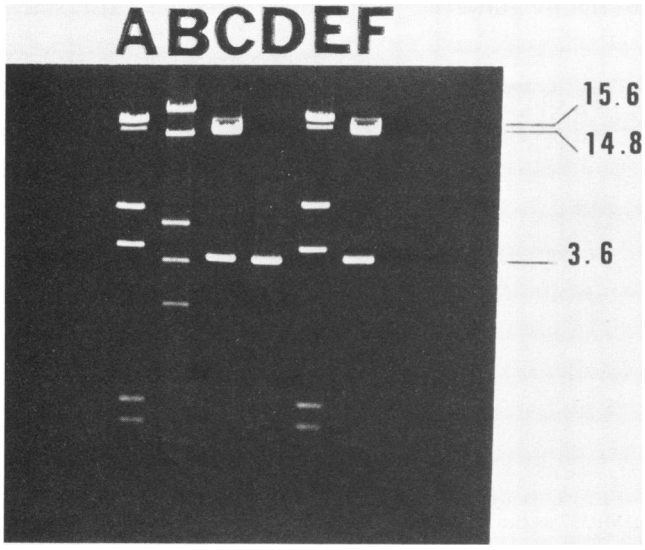

FIG. 7. Comparison of pEML159, pPSA122, and pBR325 after restriction endonuclease digestion with *Hin*dIII. Lanes: (A, E) molecular weight marker, lambda DNA; (B) pEML159; (C, F) pPSA122; (D) pBR325. The cloned fragment in pPSA122 expressing activity with radiolabeled 2,4-D is approximately 14.8 Mdal (C, F). This corresponds to fragment two (second largest) in the *Hin*dIII digestion of pEML159 (B).

pJMP397 is easily transferred to *E. coli* HB101, using the mercury resistance marker, but pEML159 does not transfer by either transformation or conjugation into strain HB101.

To construct a hybrid plasmid containing the cloned gene(s) responsible for 2,4-D degradation, pEML159 was digested with *Hin*dIII and each of the fragments was ligated to linear (by *Hin*dIII digestion) pBR325. Recombinant plasmids were selected by insertional inactivation of the tetracycline locus.

To detect activity from cloned fragments encoding 2,4-D degradation, an assay was developed based on the release of $^{14}CO_2$ from 2,4-D labeled in the acetate residue. Figure 6A shows the relative activities of $^{14}CO_2$ release by *Alcaligenes* sp. which grow on 2,4-D (EML159) and by a plasmid-cured derivative which cannot utilize 2,4-D (JMP228). The release of $^{14}CO_2$ is linear over the exposure time and the reaction kinetics are dramatically greater with the plasmid-containing strain EML159.

Since genetic manipulations involving cloning and transformation would involve *E. coli*, we were concerned with the initial poor level of $^{14}CO_2$ evolution exhibited by *E. coli* JMP397 which carries pJP4, expressing 2,4-D-biodegrading activity. Results in Fig. 6B reveal that, when the $^{14}CO_2$ assay was carried out in transformation buffer to increase cell permeability, 3,000 cpm of $^{14}CO_2$ were released by strains of *E. coli* which carry pJP4. The negative control HB101 released only 150 cpm of background counts. When the proper plasmid DNA fragment was cloned into the high-copy vector plasmid pBR325 and transformed into HB101

(PSA122), nearly 30,000 cpm of activity as $^{14}CO_2$ was detected.

Plasmid PSA122 contains a fragment of 14.8 Mdal from pEML159. A comparison of the restriction endonuclease pattern of pEML159 and the fragment incorporated into pPSA122 (Fig. 7) shows that the second largest fragment is the one containing the genetic material needed to allow for the release of $^{14}CO_2$ from labeled 2,4-D. Another cloned fragment carries the gene for mercury resistance; therefore, it is not on the same *Hin*dIII fragment responsible for $^{14}CO_2$ production.

DISCUSSION

The cultures from natural water sources in this study were surprisingly uniform in their phenotypic traits, metabolism, and cell structure. Based on criteria of maximum temperature of growth, pigment formation, flagellar arrangement, and G+C content for chromosomal DNA, they can be placed in the genus *Pseudomonas* or *Alcaligenes* (5). Only flagellar arrangement clearly separates the two genera, but flagellar arrangement is difficult to ascertain in many cases since species of *Alcaligenes* have few flagella as seen by electron microscopy. When lateral flagella were seen, the isolates were considered to be *Alcaligenes* sp. Isolate EML159 is such a strain with many characteristics in common with known *Alcaligenes* sp., strains JMP228 and JMP134, provided to us from the laboratory of J. M. Pemberton in Australia. It is probably unnecessary to make a clear distinction between *Pseudomonas* and *Alcaligenes* because species from both genera are commonly found in water, soil, and sewage and are often responsible for decomposition of man-made chemicals in the environment. Pemberton and co-workers described two species of *Alcaligenes* containing plasmids conferring the ability to degrade 2,4-D. *Alcaligenes paradoxus* is phenotypically similar to our pigmented isolates and *A. eutrophus* is phenotypically similar to the citrate-utilizing, nonpigmented strains (11, 14).

Lehmicke et al. (19), reporting on most-probable-number testing for 2,4-D-metabolizing bacteria, state that higher most-probable-number values were seen when enrichment cultures were established with low levels of 2,4-D. A similar result was seen in the present study when 1 μg of 2,4-D per ml was sequentially added to enrichments for 7 days. When these enrichments were plated onto EM Noble agar, however, no pure cultures of 2,4-D-metabolizing bacteria were isolated. It may be that cometabolism of the low levels of 2,4-D occurred in enrichment flasks containing environmental water samples laden with organic material, but when these bacteria were placed on EM 2,4-D agar plates and required to use 2,4-D as a sole carbon and energy source, they were unable to do so.

Other genera have been reported to metabolize 2,4-D, often in higher concentrations than are used here (3, 20). However, we found only one phenotypic type. The one exception was a gram-positive isolate which appeared to be a *Corynebacterium* sp. by the cellular arrangement, but it lost the ability to degrade 2,4-D soon after isolation. Due to confusion or inadequate information about relatively unstudied genera such as *Alcaligenes*, the taxonomic position of other gram-negative, nonfermentative motile rods reported previously may have been misassigned.

When selected isolates were tested for degradation of chlorinated hydrocarbons, those compounds most similar in structure to 2,4-D were most completely degraded. Independent of G+C content or other taxonomic characterization, all tested isolates metabolized 2,4-DCP, 4-chlorophenol, and 2,5-dichlorophenol. The failure to degrade 2-chlorophenol was surprising and may be due to the toxicity of that compound to the isolates tested. For example, levels higher than 50 μg of 2,4-DCP per ml were toxic to these isolates.

Two major pathways have been elucidated for degradation of 2,4-D by bacteria (3, 13, 28, 32). One pathway begins with removal of the two-carbon side chain from the ring structure forming glyoxylate and 2,4-DCP. The second pathway is different in that in the first reaction the no. 6 carbon of the ring is oxidized by the addition of a hydroxyl group producing 6-OH-2,4-dichlorophenoxyacetic acid (6-OH-2,4-D) followed then by the removal of the acetate side chain forming 3,5-dichlorocatechol. All isolates were able to utilize 2,4-D as their sole carbon source and in no case did we find intermediate metabolic products accumulating in the growth medium. Knowledge of the 2,4-D degradation pathway used by EML159 was obtained when the $^{14}CO_2$ assay for 2,4-D degradation was developed. This assay relies on removal of the two-carbon side chain of 2,4-D and its further oxidation to CO_2. It was developed to screen *E. coli* HB101 transformants containing cloned DNA fragments. The products formed from 2,4-D degradation by PSA122 were identified by peak heights, using UV scanning spectrophotometry. A peak was observed with a maximum absorbance at 290.02 nm, the characteristic absorption peak of 2,4-DCP. Since genetic material coding for an enzymatic step producing 2,4-DCP was cloned into pPSA122 from EML159, the degradative pathway involves the formation of glyoxylate and 2,4-DCP and not the 6-OH-2,4-D intermediate.

Many molecular biological procedures have been developed with strains of *E. coli*. Applying those methods to noncoliform strains has presented some difficulties. We found it necessary to modify an alkaline plasmid extraction technique to obtain sufficient cell lysis for adequate DNA yields. Ten other plasmid extraction methods, including that used by workers in Australia (11, 14), were tested before adequate DNA yields were obtained. Transformation procedures used for *E. coli* strains (7) or for *Pseudomonas* species (6, 8) were unsuccessful with our strains. Conjugation between only the two most similar strains, JMP228 and EML159, was successful. Strain-specific incompatibilities may exist for these isolates that do not pose problems in more genetically defined systems. It is not likely that these strains readily pass on their plasmids to neighboring cells in the environment since even long incubation times and high cell densities failed to provide transconjugants with our isolates.

Restriction fragment patterns generated by *Eco*RI and *Hin*dIII digests were very similar with pEML159 and pJMP397. Intensity of hybridization with dot blots, identity of restriction fragments from pEML159 and pJMP397, and identical T_m values indicate that these two plasmids are 80 to 100% homologous (16, 34). The relatedness of pJMP397 (pJP4) and pEML159 is significant since the two isolates from opposite sides of the world contain apparently identical plasmids. The second (66-Mdal) plasmid in JMP397 produces the additional fragments seen in the restriction analyses (Fig. 3). The source of the additional plasmid is not known since it was not detected in JMP397 until several months after receipt of the culture. Related genera such as *Pseudomonas* have been shown to readily incorporate plasmid DNA into their chromosome and then excise a different sized plasmid (29). If this has occurred in JMP397, the additional plasmid may be a hybrid of pJP4 and chromosomal DNA. We have

seen evidence for this in several dot blots where hybridization between pJMP397 and chromosomal JMP397 was demonstrated (data not shown).

Selected purified plasmids from three other strains were also probed on the dot blot, with ^{32}P-labeled pJMP397 as the probe DNA. However, these plasmids showed no homology with the probe DNA species. Possible explanations for this are as follows: (i) 2,4-D degradative ability is encoded in chromosomal DNA in most species which metabolize 2,4-D; (ii) there are molecular differences among gene sequences responsible for the degradation of 2,4-D; (iii) plasmid DNA from other isolates contain genes for the degradative pathway where 6-OH-2,4-D is the first intermediate and the two-carbon chain is removed in the form of acetate rather than glyoxylate.

Plasmid PSA122, which contains the gene(s) for the removal of the acetate moiety from 2,4-D, has the potential to be used as a probe for detecting and enumerating 2,4-D-degrading bacteria in the environment. Its use as a forecaster of potential degradative activities could be useful in studies of environments targeted for pesticide treatment and in correlating the presence of 2,4-D-degrading bacteria to specific rates of 2,4-D biodegradation.

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