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Large Plasmids from Soil Bacteria Enriched on Halogenated Alkanoic Acids

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Four *Pseudomonas* species and two *Alcaligenes* species were isolated from soil with a capacity to grow on halogenated alkanoic acids. They were shown to contain one of five large plasmids. The plasmids had molecular weights ranging from 98,800 to 190,000. They were associated with the ability to utilize the halogenated substrates 2-monochloropropionic acid and 2-monochloroacetic acid and with resistance towards one or more of the heavy metals mercury, selenium, and tellurium. The largest plasmid, pUU204, was shown to be unstable in continuous-flow culture when the organism was supplied with succinate as the sole carbon source. The dehalogenase gene associated with pUU204 appeared to be readily transferred to an incP group plasmid, R68-45.

Microorganisms which synthesize dehalogenase enzymes can grow on halogenated alkanoic acids (HAA) as their sole sources of carbon and energy (8, 11, 20). A number of soil bacteria containing various numbers and combinations of dehalogenase isoenzymes have previously been isolated and identified (8, 9). They include isolate E2 which is a *Pseudomonas* species (or possibly an *Alteromonas* species); isolates E3 and E4 which are two different *Pseudomonas* species; isolate E6 which is another *Pseudomonas* species, probably *Pseudomonas maltophila*; and two *Alcaligenes* species designated isolates E20 and E22. These bacteria were isolated after a period of mixed-culture enrichment in closed (batch) systems, and we have previously suggested that their successful enrichment and isolation were the result of dehalogenase gene transfer and aggregation mechanisms (7). It has previously been shown that the dehalogenase activity is the organisms' growth-rate-limiting step (22), and so any mechanism which increases an organism's overall enzyme specific activity would be expected to constitute a growth rate advantage and hence a selective advantage during enrichment culture. A number of processes could lead to such an advantage, for example, through the selection of constitutive mutants, the duplication of existing dehalogenase genes, or the aggregation within one organism of different dehalogenase genes obtained from different organisms and mediated by a gene transfer mechanism. In the present case, the first mechanism has been eliminated since all isolates possessed inducible dehalogenases (8). The second mechanism has not been tested, while the last might operate through the transfer of plasmids between different microbial populations, mobilizing different dehalogenase genes onto the transferring plasmid.

Continuous-flow culture studies by Godwin and Slater (7) showed that plasmid TP120 contained in *Escherichia coli* was structurally unstable when grown under nutrient-limited conditions. The resultant mutant populations were more competitive than the parent population, displacing it from the chemostat. Similarly, Jones et al. (12) showed that the cloning vector pBR322 was lost from *E. coli* under nutrient-limited conditions but, unlike the Godwin and Slater study (7), fragmentation of the plasmid was never observed. Structural instability has also been observed in plasmid pWWO which loses a 39-kilobase (kb) segment to yield a 78-kb conjugative, cryptic plasmid, pWWO-8, which no longer possesses catabolic enzymes for the *meta* pathway of benzoate degradation (24).

In this paper we describe the isolation of five large plasmids with molecular weights of 100,000 and higher from soil bacteria able to grow on 2-monochloropropionic acid (2MCPA) and monochloroacetic acid, and we examine the stability of one of them in continuous-flow culture as a means of determining the genotype of the plasmid.

MATERIALS AND METHODS

Bacterial strains and growth. The soil bacteria were isolated and maintained as previously described (7). These included the following: *Pseudomonas* sp. strain E2; *Pseudomonas* sp. strain E3; *Pseudomonas* sp. strain E4; *Pseudomonas* sp. strain E6; *Alcaligenes* sp. strains E20 and E22; and *Pseudomonas aeruginosa* PAO1162 (*leu*$^-$ *rmo*$^-$) and *P. putida* KT2440 (*rec*$^-$ Km^r Ap^r). The last two strains were kindly provided by K. N. Timmis, University of Geneva.

For the initial plasmid screenings and for the preparation of complete plasmid samples, the organisms were grown in 50 ml of defined growth medium with 2MCPA as the carbon source at pH 7.0 in 100-ml Erlenmeyer flasks incubated at 30°C on an orbital shaker at 200 rpm (20). The organisms used to prepare plasmids for restriction endonuclease digestion were cultured in chemostats with 2MCPA as the growth-limiting substrate as previously described (8, 9). The organisms were grown at dilution rates which were approximately 20% of their maximum specific growth rates.

Growth in continuous-flow culture. Chemostat cultures were grown in glass vessels with a working volume of 1,000 ml, agitated by a magnetically coupled stirrer, and aerated with sterile, humidified air a rate of 1,000 ml min^{-1}. The temperature (30°C) was maintained with a Churchill heating unit (Churchill instruments, Uxbridge, U.K.), and fresh medium was introduced into the vessels by flow inducers (Watson-Marlow, Falmouth, U.K.). The closed culture me-

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dium described by Hardman and Slater (8) was used. Samples of the cultures were taken and diluted in 0.1 M phosphate buffer (pH 7.0), and samples (0.1 ml) were plated onto minimal medium agar plates containing either succinate or 2MCPA at a concentration of 0.5 g of carbon liter^{-1}. Viable counts were performed after incubation at 30°C for 48 h. The chemostat cultures were screened for plasmids by the method of Wheatcroft and Williams (23).

Rapid plasmid screening and plasmid sizing. The method used for plasmid screening and sizing was based on that of Eckhardt (4), using the lysozyme and sodium dodecyl sulfate mixture for gram-negative bacteria. Samples were prepared by centrifuging (Beckman Microfuge B) 0.1 to 1.5 ml of an overnight nutrient broth culture in Eppendorf tubes for 3 to 5 min. The pellets were dried and suspended in 15 μl of lysosyme mixture and incubated at room temperature for 5 min. Samples (15 μl) were loaded into the wells of a horizontal 0.7% (wt/vol) agarose gel and carefully overlaid with 25 to 30 μl of sodium dodecyl sulfate mixture. The electrophoresis buffer was 89 mM Trizma base–2.5 mM disodium EDTA–89 mM boric acid (pH 8.2). The gels were electrophoresed at 60 mA for 60 min and stained in 0.2 μg of ethidium bromide ml^{-1} for 15 min. The gels were photographed under shortwave UV light, using Polaroid type 665 film with a Polaroid Land Camera fitted with an orange and two UV filters.

Preliminary plasmid size estimates were obtained by using this procedure with the following standard plasmids of known molecular weight (in parentheses): TP113 (57,000), TP116 (144,000), TP125 (64,000), and RA1 (86,000) (all from G. Willshaw, London, U.K.); R1 (62,000 to 65,000) (D. Godwin, Warwick, U.K.); TOL (63,000) (P. A. Williams, Bangor, U.K.); and R68-45 (36,000) (B. W. Holloway, Monash, Australia). A standard curve was produced by plotting $\log_{10}$ molecular weight against $\log_{10}$ plasmid migration distance from gel origin (millimeters) and was used to estimate the size of the unknown plasmids.

Restriction endonuclease digestion of plasmids. The procedure for restriction endonuclease digestion was based on the Wheatcroft and Williams method (23). An overnight culture (40 to 50 ml) was harvested by centrifugation at 5,000 × *g* for 10 min, the pellet was suspended in 1.6 ml of solution A, and solution B (0.4 ml) was added. The mixture was then treated as previously described (23).

Samples (0.7 ml) were loaded onto 4.0-ml sucrose gradients prepared in 5.5-ml centrifuge tubes. The gradients were prepared by two cycles of freezing and thawing a 20% (wt/vol) sucrose solution in autoclaved, distilled water (2). This produced a sucrose gradient ranging from 14 to 26% (wt/vol). The loaded gradients were centrifuged at 45,000 × *g* for 1 h at 20°C, using a swingout rotor head. Samples (each of 10 drops equivalent to approximately 0.35 ml) were siphoned sequentially from the bottom of the gradient into sterile Eppendorf tubes, and the contents of each tube were mixed. Samples (15 μl) were electrophoresed on a screening gel as described above at 60 mA for 45 to 60 min. The fractions yielding the most plasmid DNA and least RNA were divided into 40-μl aliquots and stored at −20°C until required.

Restriction endonuclease digestions were carried out with *Hin*dIII and *Pst*I (Boehringer GmbH, Mannheim), *Xho*I (Bethesda Research Laboratories Ltd.), and *Eco*RI and *Bam*HI (Miles Laboratories). The enzymes were used according to the manufacturers' instructions except that a digestion time of 2 h was used routinely. Digested plasmid DNA fragment were separated by electrophoresis on a horizontal 0.7% (wt/vol) agarose gel (18 by 24 cm) prepared in 150 ml of Tris-borate buffer. The digested samples were separately loaded on the gel with *Hin*dIII-digested lambda DNA as a molecular weight standard. The samples were electrophoresed into the gel at 40 mA for 15 min, after which the gel was flooded with electrode buffer (89 mM Tris-borate buffer, pH 8.2), overlaid with a sheet of low-density polythene, and electrophoresed for 3 h. The gel was stained and photographed as described for the rapid plasmid screening method. The Polaroid negatives were used to produce enlargements from which migration distances of the fragments were determined, and, by comparison with migration distances of the known lambda DNA fragments (16, 20), their molecular weights were determined.

Plasmid curing. Overnight cultures were grown on succinate defined medium containing 0.5 g of carbon liter^{-1} in the presence of ethidium bromide at concentrations ranging from 10 to 100 μg ml^{-1}. Samples (0.1 ml) were spread on defined medium with 1.5% (wt/vol) agar with either succinate or 2MCPA (each at 0.5 g of carbon liter^{-1}) as the carbon source. Colonies growing on succinate were replica plated onto 2MCPA defined medium, and those which failed to grow were screened for the presence of plasmids as previously described.

The colonies which failed to grow on 2MCPA were also examined for dehalogenase activity. Organisms were grown in succinate defined medium overnight, transferred to 2MCPA defined medium, and assayed for chloride release as previously described (20). The same 2MCPA colonies were examined for heavy-metal resistance as described below.

Plasmid transfer experiments. The plasmid-containing strains were used as putative donors in matings with the following recipients: *Pseudomonas* sp. strain E411 (Km^r Cm^r); *Pseudomonas* sp. strain E412 (Km^r Sm^r); *Pseudomonas putida* KT2440 (*rec*$^-$ Km^r Ap^r). Samples containing 0.2 × 10^8 to 1.0 × 10^8 donor bacteria and 0.5 × 10^{10} to 2.7 × 10^{10} recipient bacteria were jointly filtered onto sterile membrane filters (0.45-μm pore size; Millipore Corp.), placed on nutrient agar (Oxoid), and incubated at 30°C overnight. The membranes were washed in sterile 0.02 M phosphate buffer (pH 7.0), and exconjugants were selected by plating samples (0.1 ml) of the resulting suspension onto defined medium containing 2MCPA at 0.5 g of carbon liter^{-1} as the sole carbon source with appropriate combinations of drugs at the following concentrations: kanamycin, 100 μg ml^{-1}; chloramphenicol, 100 μg ml^{-1}; ampicillin, 50 μg ml^{-1}; streptomycin, 750 μg ml^{-1}. Resultant colonies were purified by streak plating on the selection medium and examined on King A medium (16). The strains were screened for the presence of plasmid DNA as described above.

The introduction of plasmid R68-45 into *Pseudomonas* sp. strain E4 was achieved by mating with *P. aeruginosa* PAO8 and selecting exconjugants on defined medium containing 2MCPA at 0.5 g of carbon liter^{-1} with the following: tetracycline, 100 μg ml^{-1}; kanamycin, 100 μg ml^{-1}; and ampicillin, 50 μg ml^{-1}. The exconjugants were examined on King A medium (16) and screened for the presence of plasmids.

Determination of heavy-metal resistance. Cultures of the plasmid-containing isolates and their cured derivatives were grown overnight on 2MCPA and succinate defined media, respectively. Samples (0.1 ml) were spread onto solid succinate defined medium. A 7.0-mm-diameter well was cut into the agar, and 175 μl of a 1 or 100 mM solution of the following metals was added: mercury ($HgCl_2$), selenium (Na_2SeO_4), tellurium ($Na_2TeO_4 \cdot 2H_2O$), zinc ($ZnSO_4$), arsenate (AsO_4), cadmium ($CdCl_2$), cobalt ($CoSO_4$), copper

TABLE 1. Plasmid nomenclature and restriction endonuclease analysis of molecular weight

Strain	Plasmid nomenclature	Restriction endonuclease sizing (kb) showing the sum of digest fragments (no. of fragments produced)					Mean size (kbs) ± SD)	Mean size (megadaltons)
		*Hin*dIII	*Pst*I	*Eco*RI	*Bam*HI	*Xho*I		
Pseudomonas sp., E2	pUU202	≈230.0 (34)	ND[a]	ND	ND	ND	≈230.0	150.0
Pseudomonas sp., E3	pUU206	156.0 (31)	150.0 (37)	ND	ND	150.0 (18)	152.0 ± 3.0	98.8
Pseudomonas sp., E4	pUU204	287.4 (48)	298.9 (45)	298.4 (49)	292.2 (32)	291.2 (39)	293.6 ± 4.0	190.8
Pseudomonas sp., E47	pUU247	21.5 (3)	ND	ND	ND	27.7 (3)	24.6 ± 3.0	16.0
Pseudomonas sp., E6	pUU206	164.1 (31)	168.8 (37)	150.5 (26)	182.7 (27)	172.1 (19)	167.6 ± 10.5	109.0
Alcaligenes sp., E20	pUU220	287.0 (34)	135.5 (38)	178.7 (35)	317.1 (42)	183.1 (43)	220.3 ± 69.5	143.3
Alcaligenes sp., E22	pUU222	264.2 (41)	202.4 (45)	222.2 (45)	ND	197.2 (46)	221.5 ± 26.4	144.1

[a] ND, Not determined.

($CuSO_4 \cdot 5H_2O$), nickel ($NiCl_2 \cdot 6H_2O$), and lead [$Pb(NO_3)_2$]. The petri dishes were incubated overnight at 30°C, and the zones of growth inhibition were measured.

RESULTS

Initial plasmid screening. The rapid screening method revealed the presence of plasmids in all soil bacteria examined (Table 1). The plasmids were large, with molecular weights in excess of 100,000 and the initial screening showed that they were of substantially different sizes and occurred singly in the six isolates examined. The largest plasmid was found in *Pseudomonas* sp. strain E4 with smaller ones in *Pseudomonas* spp. strains E3 and E6. Their nomenclature is given in Table 1. Samples were examined from populations grown in either closed or continuous-flow culture and, as expected, the plasmid sizes were comparable, although it was observed that preparations from continuously grown organisms produced much sharper electrophoretic bands than those from closed culture.

Plasmid sizing and restriction endonuclease fragment patterns. The initial attempts to size plasmid pUU204 depended on a whole-plasmid method with reference to several standard plasmids of known molecular weights. However, since all plasmids described in this paper were large, small differences in electrophoretic migration distance resulted in major discrepancies in the molecular weight determinations. It was concluded that the molecular weight of pUU204 was high and greater than the largest standard plasmid used (TP116; molecular weight, 144,000). However, to obtain accurate plasmid sizes, experiments were undertaken to produce smaller DNA fragments, using restriction endonuclease digestions and subsequent electrophoresis and sizing these by reference to a known lambda DNA digestion (Fig. 1). The overall plasmid size was calculated by summation of the individual fragment sizes for each restriction endonuclease digestion (Table 1). In accord with the whole-plasmid studies, the clearest fragment banding with the least background staining was obtained with samples prepared from organisms grow in continuous-flow culture. There was one exception to this: the restriction endonuclease digests for plasmid pUU202 consistently failed to produce adequate resolution from a high level of background staining. No adequate explanations have been formulated to explain the poor behavior of this particular strain. The use of RNase-treated samples or a preparation procedure which included a high-

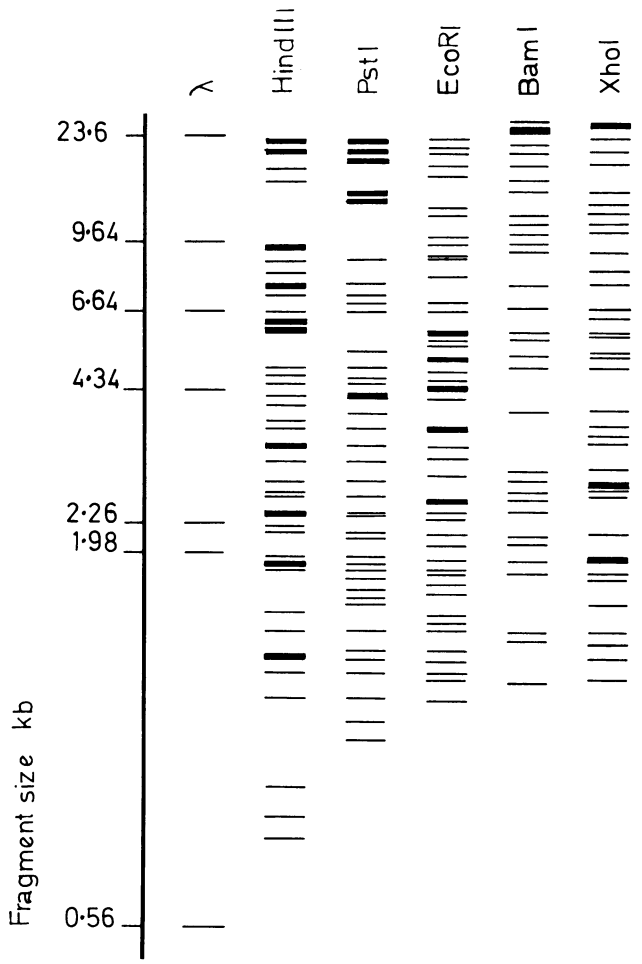

FIG. 1. Restriction endonuclease digest patterns for plasmid pUU204 from *Pseudomonas* sp. strain E4, using five restriction endonucleases. The standard is the digest pattern for restriction endonuclease *Hin*dIII digestion of lambda DNA. The thick bands represent fragments with identical or very similar molecular weights. Similar digest patterns were obtained for the other strains and the results are used in Table 1.

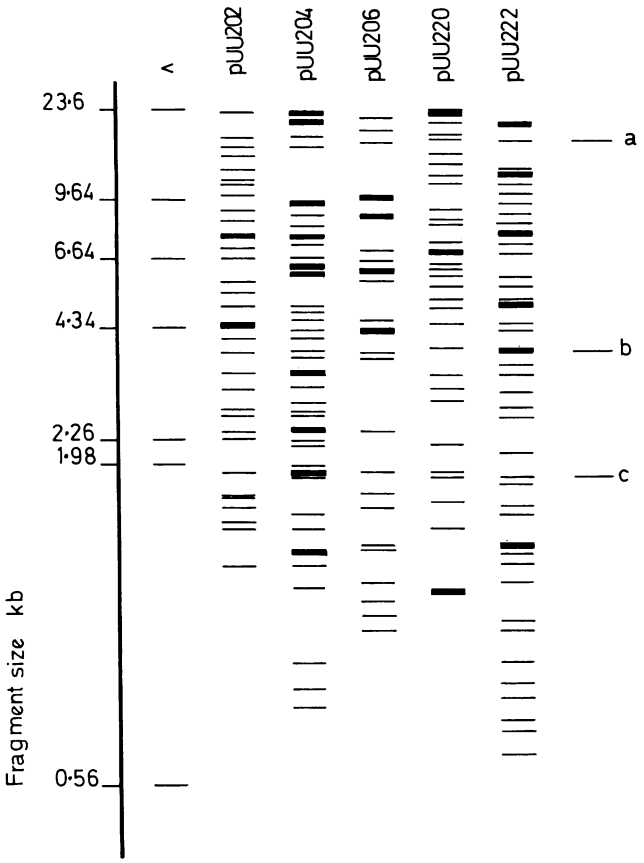

FIG. 2. Summary of the digest patterns obtained for pUU202, pUU204, pUU206, pUU220, and pUU222, using restriction endonuclease *Hin*dIII. The standard is lambda DNA also digested with *Hin*dIII. Points a, b, and c represent regions with fragments of possibly the same sizes (see text). The thick bands are described in the legend to Fig. 1.

speed centrifugation (100,000 × *g*) in sucrose gradients failed to improve the band resolution. The most satisfactory results were obtained from *Hin*dIII digestion and indicated the presence of a plasmid with a molecular weight of about 150,000 and a fragment pattern which was different from the other plasmids (Table 1).

The results showed that the molecular weight sizes differed markedly, ranging from 98,800 to nearly 200,000 (Table 1). In the case of the sizes for the plasmids from *Pseudomonas* species there was good agreement between the values determined from the different restriction endonucleases with small standard deviations. This was not seen in the case of the two plasmids analyzed from *Alcaligenes* species and at present we have no explanation for these inconsistencies.

It is clear that the plasmids differed substantially in molecular weights, suggesting that the plasmids were different. Within the limits of experimental error, however, it was possible that the plasmids possessed by the two *Alcaligenes* species and, separately, the plasmids in *Pseudomonas* sp. strains E3 and E6 were the same. Furthermore, it was possible that substantial degrees of DNA homology could exist between the different plasmids. Both of these considerations were resolved by detailed comparisons of the restriction endonuclease fragment patterns for *Pseudomonas* sp. strains E3 and E6 (Table 1; Fig. 1 and 2) and the two *Alcaligenes* species (Table 1; Fig. 2). These comparisons showed that only in the case of the independently isolated strains E3 and E6 was the fragment pattern identical, with the single exception of a small additional *Xho*I fragment detected in the preparation from strain E6 (data not shown in figures but see Table 1). It is likely that this small fragment was not visualized in the plasmid DNA digest of strain E3, and in view of the high degree of continuity with the other restriction endonucleases, these plasmids were considered to be identical and were designated pUU206. Despite the similarity of molecular weights of the plasmids from the two *Alcaligenes* species, the digest patterns showed no similarities.

The restriction endonuclease fragment patterns were complex, with large numbers of fragments being generated (Fig. 1 and 2). As anticipated, the number of fragments varied depending on which restriction endonuclease was used to cleave the DNA. The fragment numbers ranged from 49 for an *Eco*RI digest of pUU204 to 18 for an *Xho*I digest of pUU206 (Table 1). Furthermore, the fragment patterns for all enzymes used, with the exception of pUU206 from both strains E3 and E6, failed to indicate any significant homology among the five plasmids (Fig. 2). The *Hin*dIII restriction endonuclease pattern was typical, with only three regions (*a*, *b*, and *c*) which may contain fragments common to all five plasmids. However, these regions do not show exact alignment and furthermore the fragment sizes in regions *a* and *c* are sufficiently large for small differences in migration distance to make large differences in the calculated fragment molecular weights.

In one curing experiment with *Pseudomonas* sp. strain E4, using ethidium bromide, a 2Mcpa$^-$ derivative strain was isolated. This strain, designated E47, was shown to contain a small plasmid, pUU247, of molecular weight 16,000 which was not found in the parent strain (Fig. 3).

Correlation of plasmid possession with strain phenotype. An important question concerns the functions encoded by the large plasmids of these novel soil isolates. The data presented in Table 2 show that the presence of plasmids in two of the strains, *Pseudomonas* sp. strains E2 and E4, was associated with an increased resistance towards certain heavy metals. A comparison of the resistance patterns for *Pseudomonas* sp. strain E4, containing plasmid pUU204, and a cured derivative, *Pseudomonas* sp. strain E41, showed that there were significant differences in sensitivity towards mercury, selenium, and tellurium. Thus pUU204 appeared to encode resistance towards these three metals, but did not affect the response towards the seven other metals tested (Table 2). Similarly, *Pseudomonas* sp. strain E47 containing the very small plasmid pUU247 also showed greater sensitivity towards mercury, selenium, and tellurium (Table 2). In contrast, plasmid pUU202 contained in *Pseudomonas* sp. strain E2 appeared to result in resistance towards tellurium alone when compared with the plasmid-minus derivative strain E235. *Pseudomonas* sp. strains E3 and E6 demonstrated growth inhibition zones similar to that of *Pseudomonas* sp. strain E4, showing that plasmid pUU206 probably coded for resistance towards mercury, selenium, and tellurium.

For the two *Alcaligenes* species, strains E20 and E22, the zones of inhibition were the same as those for *Pseudomonas* sp. strain E4, suggesting that plasmids pUU220 and pUU222 code for mercury, selenium, and tellurium resistance. However, plasmid-free strains have not been obtained and these conclusions cannot be confirmed.

The possibility that these plasmids encoded the different dehalogenase genes was examined by curing the plasmids

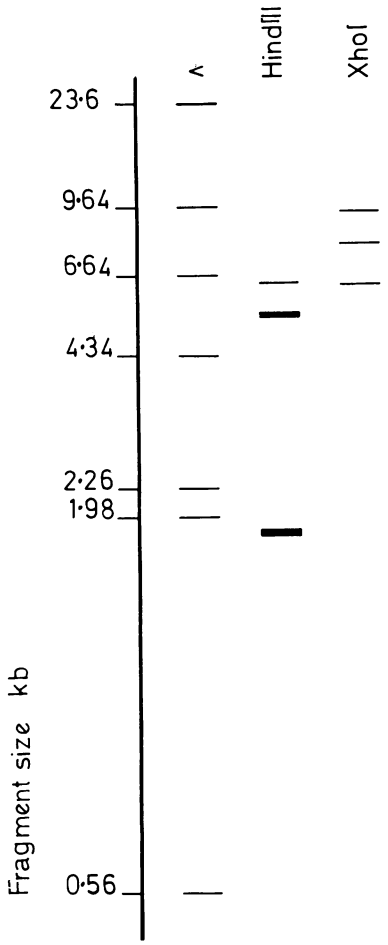

FIG. 3. *Hin*dIII and *Xho*I restriction endonuclease digest patterns for plasmid pUU247 from *Pseudomonas* sp. strain E47. The standard digest pattern is for *Hin*dIII treatment of lambda DNA. The thick bands are described in the legend to Fig. 1.

and investigating the capacity of the strains to grow on 2MCPA. The results of growing *Pseudomonas* sp. strain E4 on succinate defined medium overnight in the presence of various concentrations of ethidium bromide are shown in Table 3. These cultures were plated out onto succinate or 2MCPA defined recovery medium and indicated a substantial increase in the 2Mcpa$^-$ phenotype after treatment with ethidium bromide at concentrations in excess of 80 μg ml^{-1}. Treatment with 100 μg of ethidium bromide ml^{-1} resulted in only a threefold-lower viable organism population size when

TABLE 3. Effect of ethidium bromide on viability of an overnight culture of *Pseudomonas* sp. strain E4 plated onto a defined recovery medium with either succinate or 2MCPA as the carbon and energy source

Ethidium bromide concn (μg ml^{-1})	Viable counts (organisms ml^{-1}) on defined recovery medium containing:		% Total population able to grow on 2MCPA recovery medium
	Succinate (0.5 g of C liter^{-1})	2MCPA (0.5 g of C liter^{-1})	
0	1.70×10^9	1.05×10^9	62
40	6.00×10^8	2.63×10^8	44
80	5.30×10^8	8.80×10^5	0.002
100	5.00×10^8	2.40×10^6	0.005

compared with the untreated control culture. This compared with a 430-fold decrease in the capacity to grow on 2MCPA, at the same ethidium bromide concentration, and showed that a small fraction of the population retained the capacity to catabolize 2MCPA. Thus, there was a loss of the capacity to grow on 2MCPA which was not correlated with the general deleterious effect of growing *Pseudomonas* sp. strain E4 in the presence of ethidium bromide. Furthermore, when 100 colonies from the succinate recovery medium were individually transferred to succinate and 2MCPA media, all grew on succinate but none grew on 2MCPA. In addition, succinate-recovered isolates failed to release chloride ions after overnight succinate cultures were transferred to 2MCPA-containing defined medium. All of the putatively cured strains showed no chloride release over 24 h, whereas the parent organism showed complete breakdown of all available 2MCPA over the same time period. Finally, the possibility that the new 2Mcpa$^-$ phenotype was the result of a reversible mutation was tested by repeatedly growing the isolates on succinate and plating out high population densities (ca. 10^9 cells per plate) onto 2MCPA defined medium. No growth was observed, suggesting that the loss of the 2Mcpa$^+$ phenotype was irreversible.

A number of dehalogenase-deficient, 2Mcpa$^-$ strains were screened for the presence or absence of plasmids. All strains had lost the parent plasmid, but in one case a small plasmid, designated pUU247, of molecular weight 16,000 was isolated (Table 1; Fig. 3). Similarly, curing of the plasmid in *Pseudomonas* sp. strain E2 resulted in the nonreversible loss of the dehalogenases, but frequently the 2Mcpa$^-$ strains were found to contain a plasmid which was substantially smaller than pUU202. In the case of *Pseudomonas* sp. strain E3, no plasmids were detected in the cured strains examined.

Attempts to transfer plasmid pUU204. The various lines of evidence given in the previous sections provided initial

TABLE 2. Resistance to heavy metals by plasmid-containing and plasmid-free strains of *Pseudomonas* and *Alcaligenes* species

Pseudomonas sp. strain	Plasmid	Metal resistance-growth inhibition zones (mm)										
		Hg (1 mM)	Hg (100 mM)	Se (100 mM)	Te (1 mM)	Zn (100 mM)	As (100 mM)	Cd (100 mM)	Co (100 mM)	Cu (100 mM)	Ni (100 mM)	Pb (100 mM)
E2	pUU202	20	>30	12	0	6	0	12	15	18	16	1
E235	None	20	ND[a]	15	13	ND	ND	10	ND	ND	ND	ND
E4	pUU204	4	19	0	3	4	0	10	16	11	16	2
E41	None	15	27	10	12	4	0	ND	17	13	17	3
E47	pUU247	16	27	14	12	4	0	7	16	13	17	3

[a] ND, Not determined.

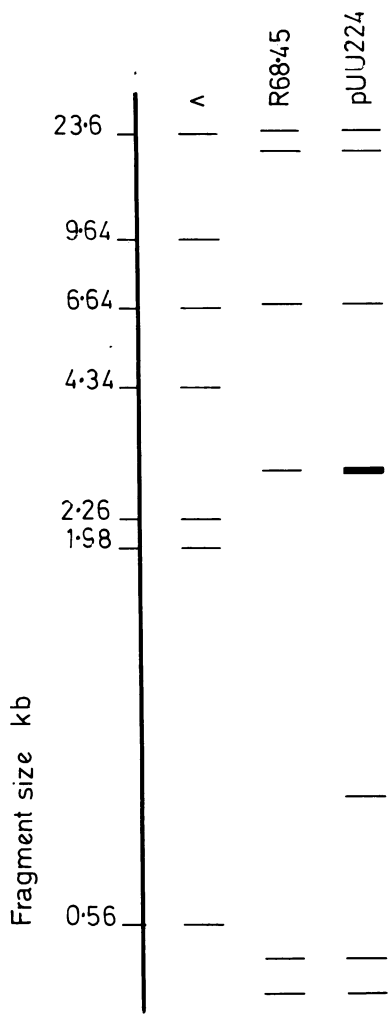

FIG. 4. Comparison of the *PstI* restriction endonuclease digest pattern for plasmid R68-45 from *P. aeruginosa* PAO8 and plasmid pUU224 from *Pseudomonas* sp. strain E4R with a *Hin*dIII endonuclease digest of lambda DNA as the standard. The thick bands are described in the legend to Fig. 1.

evidence that the dehalogenase genes and some metal resistance genes were encoded on plasmids. In attempts to prove independently that this was the case, efforts were made to transfer the plasmids to suitable recipients. Repeated attempts (with different donors and recipients and different mating conditions) failed to yield appropriate exconjugants with phenotypes which were consistent with the transfer of the dehalogenase genes. Most experiments were undertaken with *Pseudomonas* sp. strain E4 and two approaches were followed to produce 2Mcpa$^+$ exconjugants.

First, transfer experiments were performed with a cured strain of *Pseudomonas* sp. strain E4 having been made resistant to kanamycin and streptomycin, designated strain E412. In these experiments putative exconjugants were selected on defined medium containing 2MCPA, kanamycin, and streptomycin. No exconjugants were isolated and similar results were obtained when *Pseudomonas* sp. strain E411 resistant to chloramphenicol and kanamycin, *P. aeruginosa* PAO1162, or *P. putida* KT2440 were used, separately, as recipients.

Second, in the light of the apparent transfer-minus ability of these novel plasmids, attempts were made to use a known transferable plasmid, R68-45, as a vehicle for cotransferring the larger plasmid pUU204. Plasmid R68-45 was introduced into *Pseudomonas* sp. strain E4, using *P. aeruginosa* PAO8 (R68-45) as the donor. Exconjugants were obtained which grew on 2MCPA and were resistant to the drug resistances encoded by R68-45, namely, tetracycline, kanamycin, and ampicillin. The exconjugants failed to produce a green pigment on King's A medium, proving it was strain E4 and not the *P. aeruginosa* PAO8 donor. One exconjugant was designated *Pseudomonas* sp. strain E4R and shown to synthesize dehalogenase I, the enzyme known to be produced by *Pseudomonas* sp. strain E4 under batch culture conditions, as a result of its relative mobility in gel electrophoresis (8).

However, *Pseudomonas* sp. strain E4R contained only one plasmid with a molecular weight approximately the size of plasmid R68-45. Plasmid pUU204 was not found. A succinate-grown culture of *Pseudomonas* sp. strain E4 was treated overnight with ethidium bromide and 50 colonies were isolated on succinate defined medium. Only three retained the capacity to grow on 2MCPA. The 2Mcpa$^+$ phenotypes were shown to contain a plasmid designated pUU224 which was isolated and digested with the restriction endonuclease *PstI* (Fig. 4). The restriction fragments were compared with a similar digest of plasmid R68-45, but it was clear that pUU224 contained two additional fragments which in total represented an increase in molecular weight of 2,400 (or 3.7 kb). The smaller fragment was novel while the second was of the same, or very similar, size as an existing R68.45 fragment (Fig. 4). Plasmid pUU224 from *Pseudomonas* sp. strain E4R was readily transferred to *P. aeruginosa* PAO1162 at a frequency of approximately 10^{-3}, yielding exconjugants which grew on 2MCPA.

Stability of plasmid pUU204 in continuous-flow culture. Ethidium bromide treatment is known to induce chromosome lesions, and it is possible, but highly unlikely, that the curing data presented in previous sections might reflect these events rather than plasmid loss. Thus some continuous-flow culture experiments were undertaken to show that plasmid loss under nonselective and, more importantly, normal growth conditions without the effect of ethidium bromide could be correlated with the loss of a 2Mcpa$^+$ phenotype. *Pseudomonas* sp. strain E4 containing plasmid pUU204 was grown in continuous-flow culture at a dilution rate of 0.1 h^{-1}, and the plasmid's stability was investigated under succinate- or 2MCPA-limited conditions (Fig. 5). The experiment shown in Fig. 5 may be divided into three phases. In phase A, strain E4 was grown with 2MCPA as the limiting, sole carbon and energy source. During this period of 650 h of continuous growth (equivalent to nearly 95 generations), the total viable counts on either succinate or 2MCPA defined medium were the same (Fig. 5). In phase B the in-flowing medium was switched to one with succinate as the sole carbon- and energy-limiting nutrient, conditions which might be expected to select against any unnecessary capabilities, such as plasmids encoding dehalogenase functions. This was indeed the case since the total viable count as determined on succinate defined medium remained constant at approximately 10^8 organisms ml^{-1}, whereas the viable count on 2MCPA defined medium declined to undetectable levels over 530 h of continuous growth. For the remaining period of constant growth under phase B conditions, low levels of 2Mcpa$^+$ phenotypes were detected in the population but even at the highest value observed, after 1,200 h of phase B

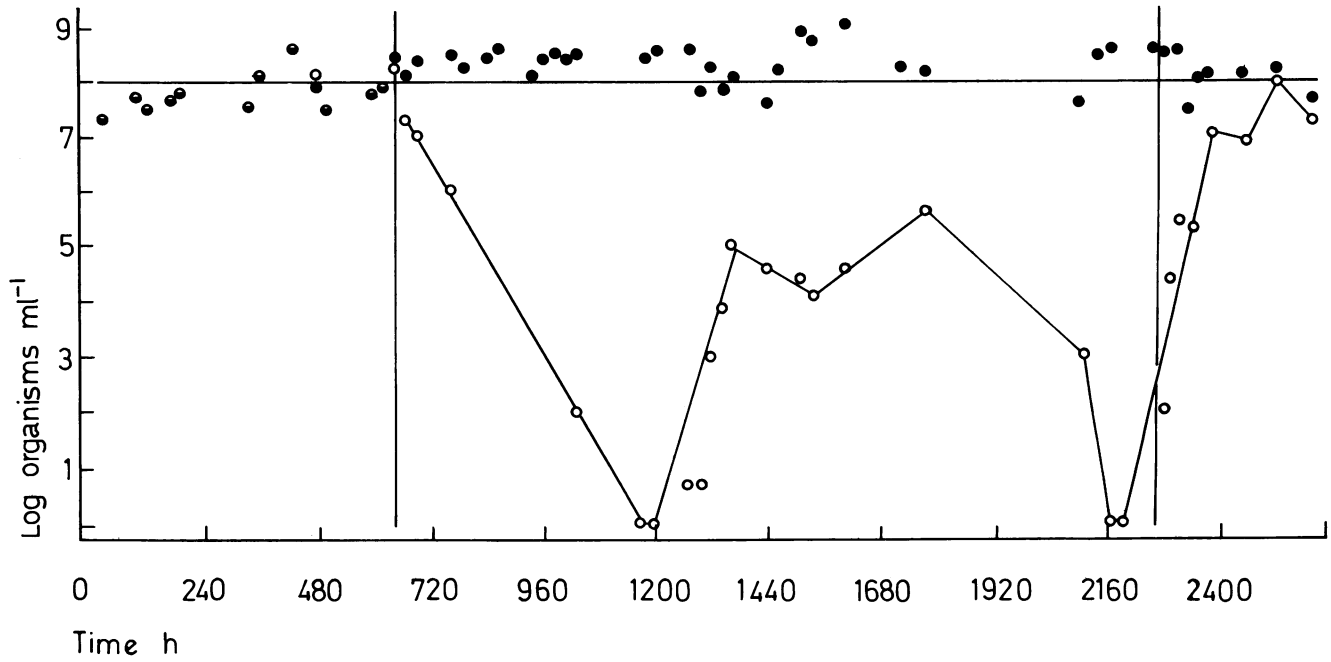

FIG. 5. Effect of carbon source on ability to utilize 2MCPA by *Pseudomonas* sp. strain E4 grown in a chemostat (see text for details). Symbols: ○, organisms growing on 2MCPA; ●, organisms growing on succinate; ◒ , shared point.

conditions, this constituted only 0.05% of the total population. After nearly 2,300 h of continuous growth (equivalent to 330 generations) the supplied medium was changed back to limiting 2MCPA to initiate phase C, and there was a rapid recovery of the 2Mcpa$^+$ phenotype population which quickly reached maximum viable count values.

The cultures during the three phases were regularly monitored for plasmid DNA. In phase A the typical pUU204 DNA was isolated, but in phase B this band of DNA was missing. When the carbon source was switched back to 2MCPA in phase C, and after full recovery of the 2Mcpa$^+$ phenotype, a smaller plasmid was detected.

DISCUSSION

The six representatives of the two genera of soil bacteria isolated after enrichment for growth on halogenated alkanoic acids were each found to contain one of a range of plasmids varying in size from about 100 to nearly 200 megadaltons. The HAA phenotype was regularly lost on growth on nonselective medium (Table 3), which suggested that perhaps it was associated with the unstable maintenance of a catabolic plasmid. The evidence presented in this paper strongly supports the view that the genes coding for the various dehalogenase genes are located on these different plasmids.

The evidence centers principally on the observation that loss of the plasmids irreversibly resulted in an inability to utilize or grow on HAA. The curing experiments showed conclusively that plasmid loss was simultaneously associated with both the loss of the dehalogenases and the resistance to certain heavy metals. In one case with *Pseudomonas* sp. strain E4, plasmid loss under nonselective conditions (i.e., growth on an unhalogenated substrate) in a chemostat showed an identical correlation without recourse to the harsh, possibly mutagenic conditions experienced in the ethidium bromide curing experiments. As expected, the chemostat evidence showed that the population maintained the dehalogenase-encoding plasmid under selective conditions (i.e., growth on 2MCPA) but it was rapidly lost when grown on succinate. The competitive exclusion of plasmid-containing organisms in favor of plasmid-free organisms under nonselective conditions is entirely consistent with the behavior of chemostat mixed cultures observed elsewhere (7, 12).

We are unable to assert categorically that these plasmids encode the dehalogenase genes and the metal resistance genes, since we have been unable to transfer the plasmids to suitable recipient strains. Despite repeated attempts under a range of environmental conditions, using both selective and nonselective recovery media, we have failed to demonstrate the concomitant transfer of the dehalogenating ability and the plasmids to suitable recipients. The plasmids are of sufficient size to expect coding for the necessary transfer genes and it was initially anticipated that they would be self-transmissible. However, we must conclude, at present, that either these plasmids do not transfer because they are transfer-minus or other factors preclude a successful transfer into a recipient. This might be due to difficulties such as host restriction, donor-recipient incompatibility, mating procedures, or simply the physical problems associated with the uninterrupted transfer of large amounts of plasmid DNA. Elsewhere, recent evidence is beginning to suggest that some large plasmids may indeed by non-self-transmissible. For example, Hooykaas et al. (10) showed that the large root-inducing plasmid (Ri plasmid) present in *Agrobacterium rhizogenes* can only be transferred in the presence of a second, mobilizing R plasmid. A nonconjugative xylene catabolic plasmid has been described in *Pseudomonas* sp. strain Pxy (6). More recently, attempts to conjugate a plasmid involved in parathion hydrolysis from *P. diminuta* to a suitable recipient failed (19). Similarly, Pickup et al. (18) concluded that two large plasmids, pWW14 (270 kb) coding for toluene degradation and pWW17 (288 kb) coding for phenylacetate degradation and mercury resistance, could not be transferred by conjugation. Thus, the HAA catabolic

plasmids reported in this paper may be another example of large non-self-transmissible plasmids. Furthermore, we have been unable to demonstrate that one of them, pUU204, can be mobilized by the presence of a promiscuous plasmid, R68-45. Also, attempts to transform suitable recipients have also proved negative (D. J. Hardman, P. C. Gowland, and J. H. Slater, unpublished data) in common with transformation attempts with other large plasmids (1, 5).

We have previously suggested that since dehalogenase activity is the growth-rate-limiting step, organisms with elevated enzyme levels will have a selective advantage (8). Thus, if the dehalogenase genes were aggregated, gene dosage effects would ensure higher dehalogenase specific activities, providing a selective growth advantage. The formation of a cluster of dehalogenase genes would clearly be facilitated if the cluster was located on a freely transmissible plasmid. As has already been remarked, these plasmids are not self-transmissible. However, it was demonstrated that at least one dehalogenase gene could be mobilized by the promiscuous incP group plasmid R68-45. This suggests that the movement and clustering of dehalogenase genes may have depended on the intervention of a readily transmissible plasmid which has not been identified. The movement of individual dehalogenase genes could be substantially enhanced if the genes were capable of transposition, possibly as transposons. There is no evidence that the dehalogenase genes on the plasmids reported in this study are located on transposons. However, the initial evidence from the experiments to mobilize plasmid pUU204 demonstrated that one dehalogenase gene was mobilized, possibly on a transposon with a molecular weight of 2,400. Elsewhere there is now substantial evidence that dehalogenase genes are located on transposons. For example, Kawasaki and colleagues (13–15) have described a dehalogenase-encoding plasmid, pU01, isolated from a *Moraxella* species and tentatively suggested that the dehalogenase gene was located on a transposon of molecular weight 3,600 (15). Beeching et al. (3) have described the transposition of the dehalogenase gene I from *P. putida* PP3 to plasmid R68-44 and initial evidence suggests that this gene is on a transposon. Thus, the original hypothesis that a number of dehalogenase genes were aggregated may still be feasible, although the reason for their clustering on nontransmissible plasmid DNA rather than smaller transmissible plasmids or the chromosome is not clear.

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LITERATURE CITED

1. **Andersen, K., R. C. Tait, and W. R. King.** 1981. Plasmids required for utilization of molecular hydrogen by *Alcaligenes eutrophus*. Arch. Microbiol. **129**:384–390.
2. **Baxter-Gabbard, K. L.** 1972. Simple method for the large scale preparation of a sucrose gradient. FEBS Lett. **20**:117–119.
3. **Beeching, J. R., A. J. Weightman, and J. H. Slater.** 1983. The formation of an R-prime carrying the fraction I dehalogenase gene from *Pseudomonas putida* PP3 using the IncP plasmid R64.44. J. Gen. Microbiol. **129**:2071–1078.
4. **Eckhardt, T.** 1978. A rapid method for the identification of plasmid desoxyribonucleic acid in bacteria. Plasmid **1**:584–588.
5. **Fennewald, M., W. Prevatt, R. Meyer, and J. Shapiro.** 1978. Isolation of Inc P-2 Plasmid DNA from *Pseudomonas aeruginosa*. Plasmid **1**:164–173.
6. **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.
7. **Godwin, D., and J. H. Slater.** 1979. The influence of the growth environment on the stability of a drug resistance plasmid in *Escherichia coli* K-12. J. Gen. Microbiol. **111**:201–210.
8. **Hardman, D. J., and J. H. Slater.** 1981. Dehalogenases in soil bacteria. J. Gen. Microbiol. **123**:117–128.
9. **Hardman, D. J., and J. H. Slater.** 1981. The dehalogenase complement of a soil Pseudomonad grown in closed and open cultures on haloalkanoic acids. J. Gen. Microbiol. **127**:399–405.
10. **Hooykaas, P. J. J., H. den Dulk-Ras, and R. A. Schilperoort.** 1982. Method for the transfer of large cryptic, non-self-transmissible plasmids: *Ex Planta* transfer of the virulence plasmid of *Agrobacterium rhizogenes*. Plasmid **8**:94–96.
11. **Jensen, H. L.** 1960. Decomposition of chloroacetates and chloropropionates by bacteria. Acta Agric. Scand. **10**:83–103.
12. **Jones, I. M., S. B. Primrose, A. Robinson, and D. C. Ellwood.** 1980. Maintenance of some Col E1-type plasmids in chemostat culture. Mol. Gen. Genet. **180**:579–584.
13. **Kawasaki, H., N. Tone, and K. Tonomura.** 1981. Purification and properties of haloacetate halidohydrolase specified by plasmid from *Moraxella* sp. strain B. Agric. Biol. Chem. **45**:29–34.
14. **Kawasaki, H., N. Tone, and K. Tonomura.** 1981. Plasmid determined dehalogenation of haloacetates in *Moraxella* sp. Agric. Biol. Chem. **45**:35–42.
15. **Kawasaki, H., H. Yahara, and K. Tonomura.** 1981. Isolation and characterisation of plasmid pU01 mediating dehalogenation of haloacetate and mercury resistance in *Moraxella* sp. B. Agric. Biol. Chem. **45**:1477–1481.
16. **King, O. E., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **44**:301–307.
17. **Phillipsen, P., R. A. Kramer, and R. W. Davis.** 1978. Cloning of the yeast ribosomal DNA repeat unit in SstI and HindIII Lambda vectors using genetic and physical size selections. J. Mol. Biol. **123**:371–386.
18. **Pickup, R. W., R. J. Lewis, and P. A. Williams.** 1983. *Pseudomonas* sp. MT14, a soil isolate which contains two large catabolic plasmids, one a TOL plasmid and one coding for phenylacetate catabolism and mercury resistance. J. Gen. Microbiol. **129**:153–158.
19. **Serdar, C. M., D. T. Gibson, D. M. Munnecke, and J. H. Lancaster.** 1982. Plasmid involvement in parathion hydrolysis by *Pseudomonas diminuta*. Appl. Environ. Microbiol. **44**:246–249.
20. **Slater, J. H., D. Lovatt, A. J. Weightman, E. Senior, and A. T. Bull.** 1979. The growth of *Pseudomonas putida* on chlorinated aliphatic acids and its dehalogenase activity. J. Gen. Microbiol. **114**:125–136.
21. **Southern, E. M.** 1979. Gel electrophoresis of restriction fragments. Methods Enzymol. **68**:152–176.
22. **Weightman, A. J., and J. H. Slater.** 1980. Selection of *Pseudomonas putida* strains with elevated dehalogenase activities by continuous culture growth on chlorinated alkanoic acids. J. Gen. Microbiol. **121**:187–193.
23. **Wheatcroft, R., and P. A. Williams.** 1981. Rapid methods for the study of both stable and unstable plasmids in *Pseudomonads*. J. Gen. Microbiol. **124**:433–437.
24. **Williams, P. A., and D. J. Jeenes.** 1981. Origin of catabolic plasmids, p. 144–147. *In* D. Schlessinger (ed.), Microbiology—1981. American Society for Microbiology, Washington, D.C.