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Molecular Sizes and Relationships of TOL Plasmids in *Pseudomonas*

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Plasmid deoxyribonucleic acid was isolated from thirteen *Pseudomonas* strains judged on genetic criteria to carry plasmids coding for the degradation of toluene and *m*- and *p*-xylenes (TOL plasmids). Most strains carried a single species, but two strains carried two size classes, and cells of a third strain contained plasmids ranging in size from 25×10^6 to 202×10^6 daltons. Some plasmids could be transformed into a *Pseudomonas putida* strain to yield Tol$^+$ progeny. Plasmids from 5 of the 13 strains were indistinguishable on the basis of size and gel pattern of fragments after endonuclease digestion.

Williams and Worsey (17) have described strains of *Pseudomonas* isolated independently from soil after enrichment culture in which *m*-toluate was the sole carbon source. These strains can also degrade toluene via benzoate and *m*- and *p*-xylene via *m*- and *p*-toluate (the Tol function). Genetic evidence was presented that in these strains this degradative function was plasmid mediated, as had previously been shown in *Pseudomonas putida* (*arvilla*) mt-2 (16, 18, 19). In this communication we (i) describe the molecular characterization of plasmids from these strains, (ii) present evidence that in most cases the presence of the plasmid is correlated with the possession of the Tol function, and (iii) describe experiments that test whether the plasmids from the different isolates are related to each other.

MATERIALS AND METHODS

Bacterial strains. The toluene- and xylene-utilizing bacteria used in this investigation have been described previously (17). The recipient for transformation, strain AC34, was kindly provided by A. M. Chakrabarty. It is an adenine-requiring derivative of *Pseudomonas putida* strain PgGl (11). Strains of *Escherichia coli* harboring the plasmids RP1 and ColE1 were obtained from G. Humphreys and N. S. Willetts, respectively.

Materials. Nitrocellulose (DHX 30/50) was obtained from the Nobel Division of I.C.I. Ltd., Stevenston, Ayrshire, Scotland. Egg-white lysozyme (grade I, lyophilized) and ethidium bromide were obtained from Sigma Chemical Co., St. Louis, Mo. All other materials were obtained from sources previously described (15).

Culture conditions and determination of growth rates. When it was necessary to select for and to ensure retention of the Tol$^+$ phenotype in bacterial strains, a minimal salts medium containing *m*-toluate as sole carbon source was used. Liquid medium was made by the addition of sterile 1 M *m*-toluate to 80 ml of 4×-concentrated M9 medium (1) to give a final concentration of 10 mM. This mixture was added to 300 ml of sterile water to which had also been added 0.5 ml of stock salts solution (3), 0.25 ml of 1 M $MgSO_4$, and 0.25 ml of 36 mM $FeSO_4$. This method of preparation causes minimal precipitation of phosphates and hydroxides of heavy metals and avoids the need to use an organic chelating agent. This medium was solidified by the inclusion of 1.5% New Zealand agar to give selective plates and slants on which stock cultures were maintained. Other prototrophic strains were maintained on minimal agar slopes containing either succinate or benzoate as the carbon source. Auxotrophic strains were maintained on L-broth agar slopes. L-broth contains 10 g of tryptone (Difco), 5 g of NaCl, and 5 g of yeast extract (Difco) per liter. Bacterial buffer contains 3 g of KH_2PO_4, 7 g of Na_2HPO_4, 4 g of NaCl, and 0.2 g of $MgSO_4 \cdot 7H_2O$ per liter.

For the isolation of degradative plasmid deoxyribonucleic acid (DNA), cell cultures were grown to stationary phase in 25 ml of selective medium containing *m*-toluate. Such cultures were inoculated into 1-liter volumes of Spizizen minimal medium (14) containing 0.2% glucose, in 2-liter conical flasks. Cell density was measured turbidimetrically with a Klett-Summerson colorimeter with a red filter. Bacterial cultures were grown in rotary shakers at 30°C for *Pseudomonas* strains and at 37°C for *E. coli* strains.

Isolation of plasmid DNA. Phage λ DNA was prepared as described (9). ColE1 and RP1 DNA were prepared from *E. coli* strains ED678 (obtained by the transfer of ColE1 into strain JC 6256 [2]) and UB 1139 (5), respectively, as described previously (15). Chloramphenicol was added to exponentially grow-

ing cultures (3×10^8 cells/ml) of the former strain to a final concentration of 60 μg/ml in order to increase the number of copies of ColE1 DNA per cell (7).

Isolation of degradative plasmid DNA. In repeated attempts, we were unable to isolate degradative plasmid DNA as covalently closed circular molecules in an ethidium bromide-CsCl density gradient after a clearing spin had been used to remove chromosomal DNA. After the finding (11) that such plasmid DNA could be isolated after removal of chromosomal DNA by alkaline denaturation and adsorption to nitrocellulose, the following modification of the method of plasmid isolation (13) was used. Using this method, a single person can prepare plasmid DNA from at least six strains simultaneously, the main work of the preparation occurring during 4 of the working days of 1 week.

One-liter batches of bacterial cultures were grown to late exponential phase (approximately 10^9 cells/ml or 180 Klett units) in Spizizen minimal medium and harvested by centrifugation at 4°C. The pellet was resuspended in 21 ml of cold sucrose solution [25% sucrose in 0.05 M tris(hydroxymethyl)aminomethane (Tris), pH 8.0] and stored in this form at −20°C. After thawing, spheroplasts were formed by adding lysozyme solution (3 ml of freshly prepared solution at 10 mg/ml in 0.25 M Tris-hydrochloride, pH 8.0) and shaking at 37°C for 2 min, followed by storage on ice. After 5 min, 13 ml of 0.25 M ethylenediaminetetraacetic acid (EDTA) solution (sodium salt, pH 8.0) was added, and after a further 5 min the cells were lysed by adding 27 ml of an aqueous solution of 2% Triton X-100, 0.05 M Tris-hydrochloride (pH 8.0), and 0.0625 M EDTA. After allowing up to 1 h for lysis, the DNA in the resulting viscous solution (approximately 60 ml) was sheared by slow passage through the Luer nozzle of a 50-ml disposable syringe for 45 to 60 s. The sheared lysate was denatured (to pH 11.5) by the addition of 2 ml of freshly made 4 N NaOH solution (or a solution that had been stored in a stoppered bottle) while stirring with both a magnetic follower and glass rod. When the mixture appeared homogeneous, the glass electrode of a pH meter was used to ascertain the homogeneity of pH (i.e., that there were no pockets of extreme pH). The solution was held at pH 11.5 for 60 s while being mixed gently; the pH was then rapidly restored to between 8.0 and 8.5 by the addition of 25 ml of saturated (at room temperature) Tris-hydrochloride solution, pH 7.0. The single-stranded DNA from denatured chromosome and open circular plasmid molecules was removed by adsorption to nitrocellulose. Approximately 20 g (wet weight) of nitrocellulose, which had been ground to fibrous mats with a porcelain mortar and pestle and washed extensively with distilled water, was sprinkled into the DNA solution and gently rotated with it for 1 h at 4°C. The nitrocellulose with the associated single-stranded DNA was removed by centrifugation at 5,000 rpm for 10 min at 4°C; this extraction step was then repeated by a further addition of nitrocellulose to the supernatant. After centrifugation, each solution was filtered through glass wool into a polycarbonate centrifuge bottle, underlayered with 4 ml of saturated CsCl (at room temperature) in TES buffer (0.05 M Tris-hydrochloride, 0.005 M EDTA, and 0.05 M NaCl, pH 8.0), and centrifuged (for 15 to 20 h) at 18,000 rpm ($32,000 \times g$ at r_{av}) in a fixed-angle type 21 rotor (Beckman-Spinco) at 4°C after the centrifuge bottle was completely filled by careful overlayering with distilled water. After gentle aspiration of the upper 90 ml, the lowest 9 ml of each tube was collected, gently mixed, and filtered through a glass wool plug into polyallomer centrifuge tubes. Solid CsCl was added to bring the refractive index to 1.402, and the tubes were each filled to within 1 cm of the top with CsCl solution of refractive index 1.402. Last, 0.2 ml of ethidium bromide solution (20 mg/ml in water) was added and gently mixed with the tube contents by inversion. From this step onward, the tubes were protected from light whenever possible to minimize light-activated dye nicking of covalently closed DNA molecules. The centrifuge tubes were overlayered with liquid paraffin and centrifuged at 40,000 rpm ($100,000 \times g$ at r_{av}) for 40 h in a fixed-angle type 50 Ti rotor (Beckman-Spinco) at 15°C. The DNA banded within the density gradient to form (i) an upper viscous band comprising linear chromosomal and open circular plasmid DNA and (ii) a lower band comprising covalently closed circular plasmid DNA. These were apparent under long-wavelength ultraviolet illumination and, if present in high yield, were also visible against a white background in daylight. The DNA bands were collected by aspiration into a Pasteur pipette connected to a 1-ml disposable syringe by a cut rubber teat. The upper band was removed first and discarded. When necessary, the material from the plasmid band was pooled and recentrifuged to concentrate the plasmid DNA and to obtain a purer preparation. However, it was generally found that a single ethidium bromide-CsCl density gradient centrifugation step was adequate to isolate plasmid DNA in a sufficiently pure form to allow subsequent characterization by electron microscopy and agarose electrophoresis after endonuclease digestion.

Ethidium bromide was removed from the plasmid preparation by gentle extraction with propan-2-ol (presaturated with CsCl solution) followed by dialysis against the sodium form of Dowex 50W-XB resin in 50 ml of buffer (0.8 M NaCl, 0.05 M Tris-hydrochloride, 0.01 M EDTA, pH 8.0) (13). The DNA solution was finally dialyzed against several changes of TE buffer (0.01 M Tris-hydrochloride and 0.001 M EDTA, pH 7.2) before storage at 4°C. The DNA concentration was determined spectrophotometrically, assuming that an absorbance at 260 nm of 1.0 corresponds to 50 μg/ml. Yields of 30 to 50 μg of plasmid DNA per preparation were obtained.

Transformation. A scaled-down version of a published transformation procedure (6) was used, with strain AC34 as recipient. A 50-μl amount of chilled calcium-treated cells was added to a mixture of 20 μl of plasmid DNA, containing approximately 0.5 μg of DNA, and 5 μl of 0.5 M $CaCl_2$ at 0°C, mixed, and stored on ice for 60 min. After the heat-pulse step (42°C, 5 min), the cell suspension was kept on ice for at least 30 min, after which a 10-μl sample was removed with a micropipette, mixed with 0.1 ml

of bacterial buffer, and plated out onto selective medium, containing 10 mM *m*-toluate and adenine (50 μg/ml), for the detection of transformants.

The remainder of the cell suspension was mixed with 10 volumes of L-broth and shaken at 30°C for 3 h. The cells were then harvested by centrifugation and resuspended to the same density in bacterial buffer. Samples of 0.1 and 0.2 ml were plated out onto the selective agar plates. Viable counts were made at all stages of the transformation procedure on L-broth agar.

Electron microscopy. Plasmid DNA was visualized by the formamide method (8).

Restriction endonuclease digestion of plasmid DNA. Digestion was carried out in silicone-coated (Repelcote; Hopkins and Williams) small tubes (12.5 by 50 mm) sealed with Parafilm (Gallenkamp) at 37°C for up to 4 h, depending upon the activity of the enzyme preparation. DNA preparations in TE buffer containing approximately 1 μg of DNA were each dried down to 20 μl in a vacuum desiccator over concentrated H_2SO_4 at a reduced pressure of 12 mm of Hg. The digestion mixture was adjusted to 10 mM Tris-hydrochloride (pH 7.5), 10 mM 2-mercaptoethanol, 100 mM NaCl, and an excess of 10 mM $MgCl_2$ over the EDTA concentration. Endonuclease *Eco*RI, prepared by the method of R. Yoshimori (Ph.D. dissertation, University of California, San Francisco, 1971) in 50% glycerol, was added in requisite amounts, usually 1 to 2 μl, to the digestion mixture. Endonuclease *Xma* I, purified from *Xanthomonas malvacearum* and which cleaves the DNA sequence

5′ - C↓C C G G G - 3′
3′ - G G G C C↑C - 5′

(S. Endow and R. J. Roberts, manuscript submitted for publication), was used in a similar manner.

After incubation, the reaction was terminated by heating in a water bath at 70°C for 10 min, after which the tubes were rapidly chilled in ice water; this prevents the cohesive ends of λ DNA from reannealing. The contents of each tube were mixed with 5 μl of loading mixture, comprising 10% Ficoll (Pharmacia) and 0.04% bromophenol blue, on polythene sheeting, and the liquid volume was reduced to about 10 μl by evaporation in a vacuum desiccator before the concentrated solution was loaded into the sample wells of the agarose slab gel.

Agarose gel electrophoresis. Electrophoresis was carried out on vertical slab gels (1% agarose) as previously described (15) except that ethidium bromide was omitted from the gel buffer and reservoir buffer and the lowest 3 cm of the slab gel was formed from 3% agarose in electrophoresis buffer to prevent gel slippage. After electrophoresis at a constant voltage of 150 V for 15 to 20 h, when the blue tracking dye had traveled about three-quarters of the length of the plate, the agarose gel was removed from the glass plates and stained for 30 min in aqueous ethidium bromide solution (2 μg/ml), after which the gel was washed in water for up to 3 h before photography. The gels were photographed against a black polythene sheeting background under short-wavelength ultraviolet illumination through a ×4 red filter onto Ilford FP4 film.

Densitometry. Densitometric scans of photographic films of gels were made on a MK.III double-beam recording microdensitometer (Joyce, Loebl and Co. Ltd., Gateshead, England).

RESULTS

Isolation and size determination of plasmid DNA. The procedure described above yielded from each of the strains examined DNA that gave a discrete band of presumptive plasmid DNA in ethidium bromide-CsCl density gradient centrifugation. This DNA was examined by electron microscopy. The preparations from most strains contained only a single size class of circular molecules. From each such preparation six molecules were measured, using ColE1 or RP1 molecules as internal standards (Table 1). Strains MT1 and MT3 yielded plasmids that fell into two size classes, and another strain, MT14, gave circular molecules of a number of different sizes (Table 2). The molecules from MT15 and MT19, and some of those from MT14, were very large. It has so far not been possible to obtain circular molecules from the presumptive plasmid DNA preparation from strain MT20 under these conditions. The possible reasons for this will be discussed in another communication.

Gel electrophoresis of endonuclease digests of plasmid DNA. Since several of the plasmids were very similar in size (about 77 megadaltons [Md]), it was possible that they were related. One test of identity is by digestion with site-specific endonucleases, followed by electrophoresis on agarose gels. Similar patterns of frag-

TABLE 1. *Sizes of plasmid molecules observed*[a]

P. putida strain	Plasmids size (Md ± standard error)	Name of plasmid
mt-2	78.1 ± 1.1	pWW0
MT16	81.3 ± 2.5	pWW16
MT17	75.0 ± 0.6	pWW17
MT18	75.6 ± 0.9	pWW18
MT21	74.4 ± 0.8	pWW21
MT1	52.0 ± 0.4	pWW1
	4.8 ± 0.1	pWW2
MT3	79.9 ± 0.8	pWW3
	4.0 ± 0.1	pWW4
MT5	103.3 ± 4.9	pWW5
MT12	75.1 ± 1.4	pWW12
MT13	75.7 ± 1.4	pWW13
MT14	25–202	
MT15	169.1 ± 2.6	pWW15
MT19	151.5 ± 2.7	pWW19

[a] Grids were first made without any internal standard. Then new grids were made including ColE1 (taken as 4.2 Md [4]) or RP1 (determined using ColE1, as 38.9 ± 0.5 Md) as internal standards.

TABLE 2. *Size distribution of circular plasmid molecules from strain MT14*[a]

Size class (Md ± standard error)	No. of molecules involved
25.2 ± 0.5	6
39.5 ± 0.8	8
60	3
68	3
76	1
202.5 ± 1.5	—[b]

[a] All such molecules on a given grid were photographed and measured. ColE1 was used as an internal standard.

[b] A total of 20 very large molecules were identified. The value given is for the seven, chosen at random, that were measured.

ments are evidence of relatedness (15). The results of such experiments with endonuclease *Eco*RI are presented in Fig. 1.

By this criterion, the plasmids with sizes of about 77 Md fell into two groups, typified by those carried by strains *P. putida* mt-2 and MT12 (Fig. 1). Digestion of the first set with another endonuclease, *Xma* I, also gave indistinguishable patterns. We conclude that this type of plasmid is widely distributed in nature. Strains MT12 and MT13 were isolated from the same enrichment culture, but could be distinguished by their morphology (17). Therefore a given plasmid can be present in different and distinguishable *Pseudomonas* species.

The plasmid DNA from strains MT1, MT3, MT5, and MT19 gave unique patterns, as expected from the size determinations, but seemed to have common bands (Fig. 2). The possibility that these plasmids are related is currently being tested by heteroduplex and annealing methods.

Densitometry traces of the gels showed that the *Eco*RI digest of the *P. putida* mt-2 plasmid DNA gave about 29 bands, on the assumption that peaks representing twice the unit amount of DNA represented coincident bands (Fig. 3). However, it is not excluded that some such double-large peaks represent duplications of particular regions. Comparison of the mobilities of these fragments with those of fragments of known size derived from a concomitant digestion of phage λ DNA indicated that the sum of the sizes of the fragments was about 75 Md. Since this was very similar to the size of the plasmid DNA as determined by electron microscopy, we conclude that this indeed represents a single plasmid species. Similar arguments apply to the other plasmid preparations containing a single size class.

Correlation of plasmid species with Tol⁺ phenotype. Transformation provides the most rigorous demonstration that a given plasmid species is responsible for a given phenotype. We have transformed plasmid DNA from *P. putida* mt-2 into a genetically marked plasmid-free strain, AC34. Our frequency of transformation (equivalent to 30 colonies per μg of DNA) was lower than that observed for the TOL plasmid

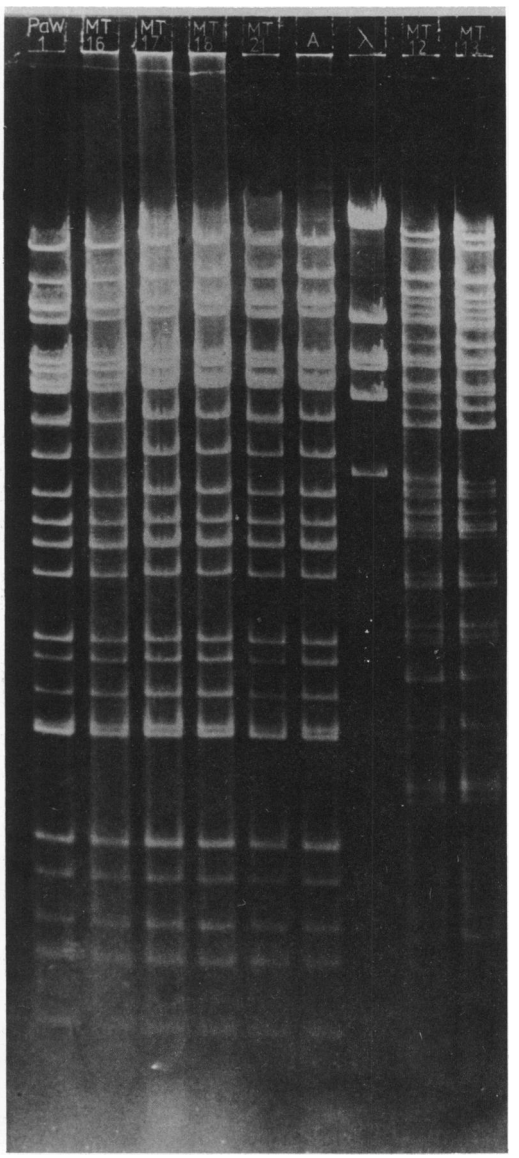

FIG. 1. *Agarose gel electrophoresis of fragments of plasmid DNA generated by EcoRI digestion. The track marked "A" is of plasmid DNA from a transformant clone (see text), and a λ digest is included to calibrate the sizes of the fragments.*

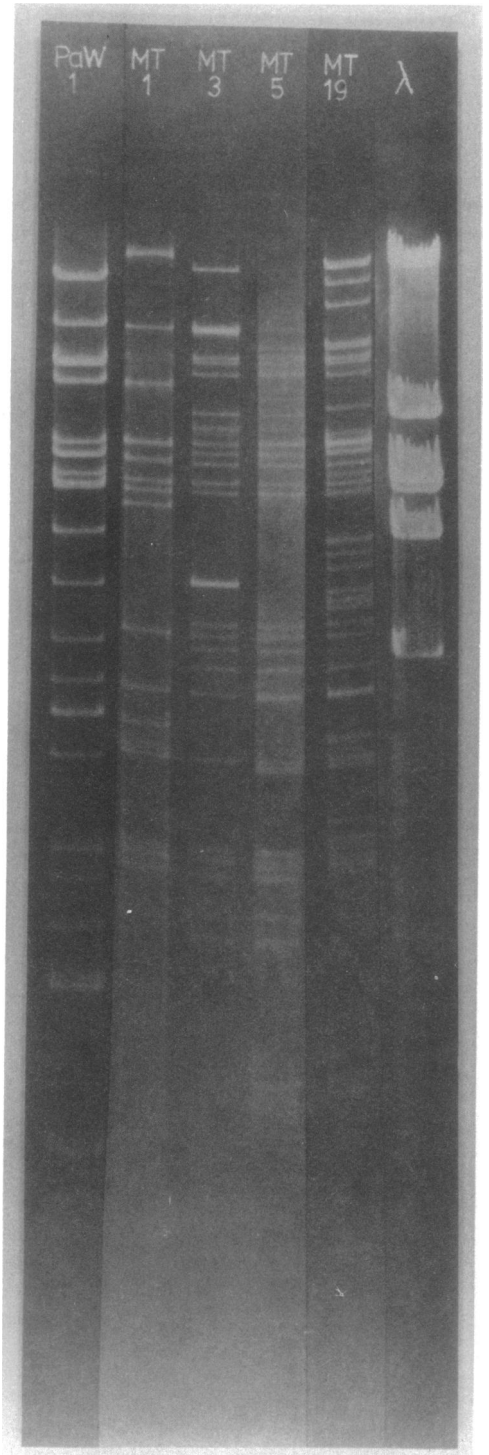

FIG. 2. *Agarose gels of EcoRI digests of unique species of plasmid DNA from Tol⁺ strains, together with those from λ and PaW1 plasmid DNA.*

by A. Chakrabarty (personal communication). This lower frequency was most probably due to the absence of conjugation after transformation under our conditions. All transformants tested were Tol⁺, conjugally proficient, and auxotrophic for adenine. The Tol⁺ progeny obtained from three independent transformants contained plasmid DNA that was indistinguishable after digestion and gel electrophoresis from the plasmid DNA of *P. putida* mt-2 (Fig. 1). Electron microscopy of these plasmid species showed that by this criterion too the plasmid was indistinguishable from that of the parental strain. Simultaneously, attempts were made to prepare plasmid DNA from strain AC34, the recipient strain in these experiments. No plasmid band was detectable in ethidium bromide-CsCl density gradients after several attempted isolations from this strain. Transformation experiments with plasmid DNA from strain MT17 gave similar results. These plasmid species can therefore be referred to unequivocally as TOL plasmids.

With plasmid species other than those of the *P. putida* mt-2 type, we have not yet been able to get transformation. However, in those cases where only one plasmid species is present, we can assign the TOL function to those plasmids on genetic criteria. Moreover, strains MT3-B1, MT15-B1, MT16-B1, MT19-B1, MT20-B1, and MT21-B1, which are Tol⁻ segregants (17), were found to be plasmid-free. The question of whether both plasmids in strains MT1 and MT3 are involved in the TOL-specified function is still open. The significance of the heterogeneity of plasmid population in strain MT14, and of the molecular basis of the TOL-specified function in strain MT20, will be the subjects of further reports.

DISCUSSION

We have shown that in at least some of the soil pseudomonads we have studied, the TOL function is plasmid borne. It is striking that in a number of independently isolated strains (*P. putida* mt-2, MT16, MT17, MT18, and MT21), the TOL plasmids must be very closely related. This group could also be distinguished from the other Tol⁺ strains studied here by genetic and physiological criteria (17). These results indicate strongly that some plasmids may be very widely distributed in nature. Other plasmids are superficially different, although some molecular relationships may be revealed by annealing or heteroduplex experiments.

It is worth noting that the original TOL⁺ strain, *P. putida* (*arvilla*) mt-2, was apparently isolated in Japan (10), whereas the others

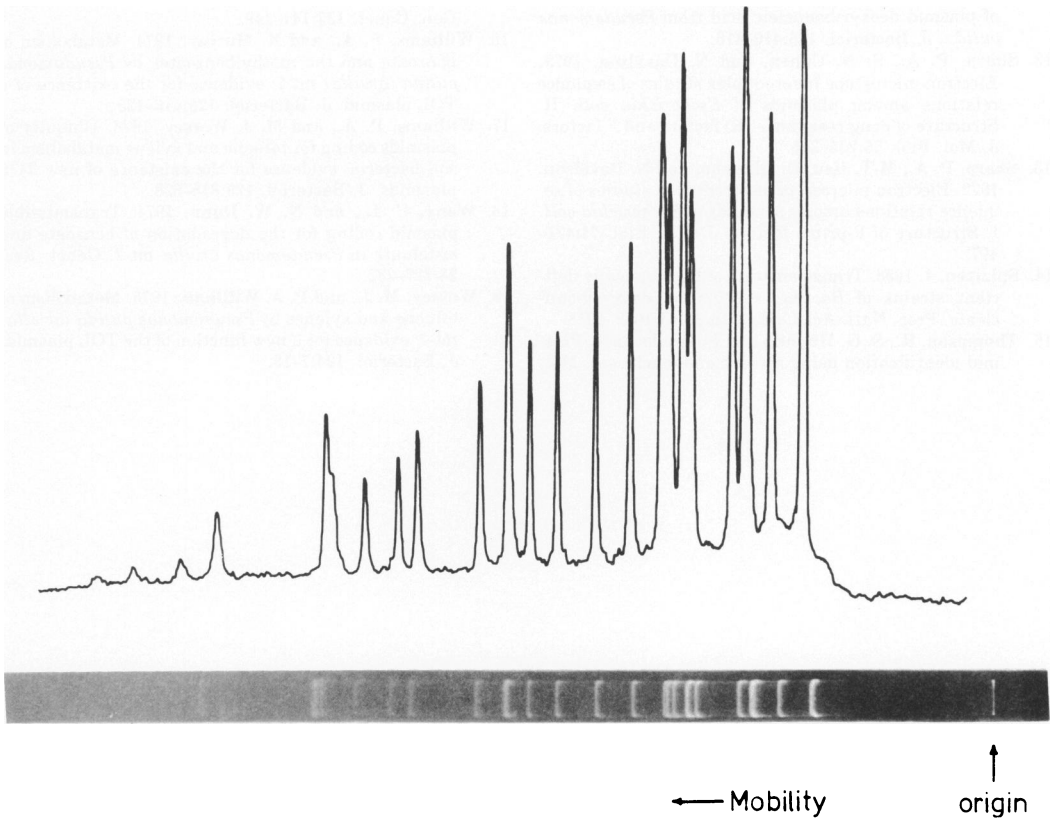

FIG. 3. *Densitometry trace of endonuclease-derived fragments of plasmid pWW0.*

of the series were isolated in Wales. The analogy with the R factors R1, R6, and R100 is striking. These R factors were originally isolated from hospital isolates in England, Germany, and Japan, respectively, but are clearly related (12). Other examples are beginning to emerge in which a group of related plasmids are widely distributed. However, the ubiquity of particular types of R factors could result from disseminating of their host strains as human commensals. Such an explanation seems unlikely for degradative plasmids of soil bacteria.

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