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# Degradation of Phenol and *m*-Toluate in *Pseudomonas* sp. Strain EST1001 and Its *Pseudomonas putida* Transconjugants Is Determined by a Multiplasmid System

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**The utilization of phenol, *m*-toluate, and salicylate (Phe$^+$, *m*Tol$^+$, and Sal$^+$ characters, respectively) in *Pseudomonas* sp. strain EST1001 is determined by the coordinated expression of genes placed in different plasmids, i.e., by a multiplasmid system. The natural multiplasmid strain EST1001 is phenotypically unstable. In its Phe$^-$, *m*Tol$^-$, and Sal$^-$ segregants, the plasmid DNA underwent structural rearrangements without a marked loss of plasmid DNA, and the majority of segregants gave revertants. The genes specifying the degradation of phenol and *m*-toluate were transferable to *P. putida* PaW340, and in this strain a new multiplasmid system with definite structural changes was formed. The 17-kilobase transposable element, a part of the TOL plasmid pWWO present in the chromosome of PaW340, was inserted into the plasmid DNA in transconjugants. In addition, transconjugant EST1020 shared pWWO-like structures. Enzyme assays demonstrated that *ortho*-fission reactions were used by bacteria that grew on phenol, whereas *m*-toluate was catabolized by a *meta*-fission reaction. Salicylate was a functional inducer of the enzymes of both pathways. The expression of silent metabolic pathways of phenol or *m*-toluate degradation has been observed in EST1001 Phe$^-$ *m*Tol$^+$ and Phe$^+$ *m*Tol$^-$ transconjugants. The switchover of phenol degradation from the *ortho*- to the *meta*-pathway in EST1033 also showed the flexibility of genetic material in EST1001 transconjugants.**

In several representative strains of the genus *Pseudomonas*, the genetic information required for the degradation of different aromatic hydrocarbons is carried by metabolic plasmids. A number of independent plasmids which determine the degradation of toluene, xylene, salicylate, naphthalene, and their various derivatives have been isolated (6–8, 13, 14, 36–38). The best studied pseudomonads of this group harbor a single metabolic plasmid (9, 19, 33, 34). Multiplasmid bacterial strains that carry several metabolic plasmids can be constructed, but they are unstable (2, 17, 31). The plasmid content has been studied in detail only in some strains, whereas the plasmids of the parent strains have been well characterized (11). Plasmid transfer to the new host may lead to plasmid dissociation, recombination with the resident plasmid, or chromosome-mediated rearrangements in plasmid DNA (7, 10, 11, 18, 23). Such changes may often result in the switching off of metabolic clusters or in the expression of silent genes (7, 18, 30, 34).

Characterization of two of the best studied metabolic plasmids, NAH/SAL and TOL, revealed common features in their structure and regulation (40). Detailed molecular analysis of the TOL plasmid pWWO has revealed the transposable nature of the metabolic cluster in pWWO (35). The integration of the 56-kilobase (kb) segment of TOL DNA into the host chromosome may result in the deletion of 39 kb of TOL DNA (27). The flanking regions form a continuous genetic element of approximately 17 kb. The latter has been retained in the chromosome of *Pseudomonas putida* PaW340 and some other isogenic strains (27, 28, 35). Recently, it has been demonstrated that the 56-kb segment of pWWO and its 17-kb derivative are transposons Tn*4651* and Tn*4652* (32).

Some phenol-utilizing pseudomonads harbor TOL, NAH/SAL, or related plasmids. For example, *Pseudomonas* sp. strain NCIB9816 carries a NAH plasmid, pWW60-1 (7, 15), and *Alcaligenes eutrophus* 345 carries a TOL plasmid, pRA1000 (22). The introduction of the TOL plasmid pWWO into a 3-chlorobenzoatenzoate-degrading strain, *Pseudomonas* sp. strain B13, has been used to expand the substrate range of the host pathway for the catabolism of halogen-substituted phenolic compounds, but it still requires structural rearrangements in the TOL plasmid (11). It has also been reported that the presence of the TOL plasmid pWWO facilitates the survival of phenol-utilizing bacteria at higher concentrations of phenol (39). However, until now no metabolic plasmid has been reported which allows phenol degradation.

In this report we characterize a natural multiplasmid system which determines the degradation of phenol, *m*-toluate, and salicylate in *Pseudomonas* sp. strain EST1001. We show that the 17-kb transposable element of the TOL plasmid pWWO takes part in the generation of a new multiplasmid system for the degradation of phenol and *m*-toluate in *P. putida* PaW340. The switchover of phenol degradation from the *ortho*- to the *meta*-pathway in multiplasmid transconjugants is demonstrated.

## MATERIALS AND METHODS

**Bacterial strains.** The bacterial strains used in this study are listed in Table 1. *Pseudomonas* sp. strain S13 was isolated in 1976 by P. A. Williams from soil samples after culture enrichment with *m*-toluate as the sole source of carbon and energy. We found that this culture spontaneously lost the ability to grow on *m*-xylene during passages on minimal salts agar containing 5 mM *m*-toluate. *Pseudomonas* sp. strain S13 *m*Xyl$^-$ derivative was designated EST1001.

**Media and culture conditions.** L broth (24) was used as a complete medium, while M9 medium (1) supplemented with a solution of microelements (4) was used as a minimal medium. Final concentrations of *m*-toluate, benzoate, succinate, and salicylate were 5 mM; the final concentration of

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TABLE 1. Bacterial strains used in this study

| Strain designation | Relevant phenotype[a] | Parent | Reference or source |
|---|---|---|---|
| *Pseudomonas* spp. | | | |
| S13 | $m$Xyl$^+$ $m$Tol$^+$ Phe$^+$ Sal$^+$ | | P. A. Williams (Wales, United Kingdom) |
| EST1001 | $m$Xyl$^-$ $m$Tol$^+$ Phe$^+$ Sal$^+$ | S13 | Spontaneous |
| EST1002 | $m$Xyl$^-$ $m$Tol$^+$ Phe$^-$ Sal$^-$ | EST1001 | Cultivation in L broth |
| EST1003 | $m$Xyl$^-$ $m$Tol$^+$ Phe$^+$ Sal$^-$ | EST1001 | Cultivation of L broth |
| EST1004 | $m$Xyl$^-$ $m$Tol$^-$ Phe$^+$ Sal$^-$ | EST1001 | Cultivation in phenol minimal medium |
| *P. putida* | | | |
| PaW1 | $m$Xyl$^+$ $m$Tol$^+$ Phe$^-$ Sal$^-$ | | (36) P. Broda (Scotland, United Kingdom) |
| PaW85 | $m$Xyl$^-$ $m$Tol$^-$ Phe$^-$ Sal$^-$ | PaW1 | (5) C. J. Duggleby (Scotland, United Kingdom) |
| PaW340 | $m$Xyl$^-$ $m$Tol$^-$ Phe$^-$ Sal$^-$ Sm$^r$ Trp$^-$ | PaW85 | (16) C. J. Duggleby (Scotland, United Kingdom) |
| EST1010 | $m$Xyl$^-$ $m$Tol$^+$ Phe$^-$ Sal$^-$ Sm$^r$ Trp$^-$ | PaW340 | Conjugation with EST1001, selection for $m$Tol$^+$ |
| EST1020 | $m$Xyl$^-$ $m$Tol$^+$ Phe$^+$ Sal$^-$ Sm$^r$ Trp$^-$ | PaW340 | Conjugation with EST1001, selection for Phe$^+$ |
| EST1030 | $m$Xyl$^-$ $m$Tol$^-$ Phe$^+$ Sal$^-$ Sm$^r$ Trp$^-$ | PaW340 | Conjugation with EST1001, selection for Phe$^+$ |
| EST1033 | $m$Xyl$^-$ $m$Tol$^+$ Phe$^+$ Sal$^-$ Sm$^r$ Trp$^-$ | EST1030 | Spontaneous $m$Tol$^+$ mutant |

[a] Phenotype designations: $m$Xyl$^+$, $m$Tol$^+$, Phe$^+$, and Sal$^+$, the ability to grow on *m*-xylene, *m*-toluate, phenol, and salicylate, respectively, as a sole source of carbon; Trp$^-$, requirement for tryptophan; Sm$^r$, resistance to streptomycin.

phenol was 2.5 mM. *m*-Xylene was spotted onto the center of a sterile filter paper disk on an inverted plate. Streptomycin was used at a final concentration of 1,000 μg/ml. Tryptophan was added at a final concentration of 20 μg/ml. Bacteria were cultivated at 30°C.

**Enzyme assays.** Cells for enzyme assays were grown in 30 ml of M9 liquid medium at 30°C to the mid-exponential phase of growth. Phenol, *m*-toluate, salicylate, or succinate was added to the medium as a sole source of carbon and energy. The cell suspension was then cooled on ice before the bacteria were harvested by centrifugation. The pellet that was obtained was washed with 10 ml of 100 mM potassium phosphate buffer (pH 7.5).

To obtain cell extracts the pellet was suspended in 5 ml of potassium phosphate buffer containing 10% acetone, and then the cells were ruptured by sonication. The cell extract was centrifuged at 45,000 × *g* for 30 min. For whole-cell assays, cells were washed a second time with 10 ml of potassium phosphate buffer.

Catechol 1,2-dioxygenase (EC 1.13.11.1) was assayed as described by Hegeman (21). Catechol 2,3-dioxygenase (EC 1.13.11.2) was assayed as described by Feist and Hegeman (15). The protein concentrations were estimated by the method of Lowry et al. (25).

The uptake of oxygen by the whole-cell suspensions was determined at 30°C with a Clark oxygen electrode by the procedure of Sala-Trepat et al. (29). The reaction vessel of oxygen electrode contained 1.4 ml of 0.1 M potassium phosphate buffer (pH 7.5) saturated with air and the cell suspension in a total volume of 1.5 ml. After determination of the endogenous uptake of oxygen, 5 μmol of phenol was added to the reaction vessel and the level of phenol oxidation was calculated as the difference between endogenous uptake and endogenous respiration.

**Elimination experiments.** After 10 generations in liquid medium supplemented with *m*-toluate, salicylate, or phenol as the source of carbon and energy, the bacteria were spread onto plates with the same carbon source. A total of 100 single colonies from each plate were tested for their ability to grow on phenol, *m*-toluate, and salicylate.

**Conjugal transfer.** Conjugal matings were carried out as described by Williams and Murray (36). Transconjugants selected from phenol- or *m*-toluate-containing minimal selective plates were tested for their ability to use all three growth substrates, phenol, *m*-toluate, and salicylate (Phe$^+$, $m$Tol$^+$, and Sal$^+$ characters, respectively).

**DNA manipulations.** Plasmid DNA was isolated by the method of Hansen and Olsen (20). For restriction endonuclease digestion and $^{32}$P labeling, plasmid DNA was banded by cesium chloride-ethidium bromide centrifugation. DNA manipulations and analyses were performed as described by Maniatis et al. (26). Transformation with plasmid DNA was carried out by the method of Bagdasarian and Timmis (3). The transformation mixture was plated onto minimal medium containing *m*-toluate, salicylate, or phenol.

## RESULTS

**Substrate specificity and stability of *Pseudomonas* sp. strain EST1001.** *Pseudomonas* sp. strain EST1001 has the ability to grow on *m*-toluate ($m$Tol$^+$), phenol (Phe$^+$), and salicylate (Sal$^+$); it can grow slowly on *p*-cresol and catechol as the sole sources of carbon and energy. The culture has maintained its growth characteristics while stored on L agar for 10 years.

The stability of the $m$Tol$^+$, Phe$^+$, and Sal$^+$ characters of EST1001 depends on the bacterial growth substrate. The $m$Tol$^+$ phenotype was stably maintained in *m*-toluate minimal medium and in L broth, while Phe$^-$ colonies and Sal$^-$ colonies appeared at a frequencies of 3 to 7% and 97 to 100%, respectively (Table 2). On the other hand, after the growth of bacteria during 7 to 10 generations on phenol- or salicylate-containing minimal medium, both Sal$^+$ and Phe$^+$ characters were maintained, while the frequency of the loss of the $m$Tol$^+$ phenotype was 1 to 5%.

The majority of Phe$^-$, $m$Tol$^-$, and Sal$^-$ eliminants gave spontaneous revertants when selected for the lost phenotypic character, indicating that the genetic information for the expression of these phenotypic markers has not been lost in bacteria.

TABLE 2. Frequency of loss of $m$Tol$^+$, Sal$^+$, and Phe$^+$ characters in *Pseudomonas* sp. strain EST1001 growing on various growth substrates

| Growth substrate | Frequency (%) of loss of phenotypic markers | | |
|---|---|---|---|
| | $m$Tol$^+$ | Phe$^+$ | Sal$^+$ |
| L broth | 0 | 7 | 100 |
| *m*-Toluate | 0 | 3 | 97 |
| Salicylate | 1 | 0 | 0 |
| Phenol | 5 | 0 | 0 |

TABLE 3. Conjugal transfer of *m*Tol$^+$, Sal$^+$, and Phe$^+$ characters from *Pseudomonas* sp. strain EST1001 to *P. putida* PaW340

| Selective carbon source | Transfer frequency[a] of transconjugants with the following phenotypes[b]: | | | |
|---|---|---|---|---|
| | *m*Tol$^+$ Phe$^-$ | *m*Tol$^-$ Phe$^+$ | *m*Tol$^+$ Phe$^+$ | Sal$^+$ |
| *m*-Toluate | $1.0 \times 10^{-3}$ | ND[c] | $1.0 \times 10^{-5}$ | $<1 \times 10^{-8}$ |
| Phenol | ND | $0.8 \times 10^{-3}$ | $2.0 \times 10^{-4}$ | $<1 \times 10^{-8}$ |

[a] Defined as the number of transconjugants per donor cell.
[b] Transconjugants selected from phenol- or *m*-toluate-containing minimal medium were tested for their ability to use all three growth substrates phenol, *m*-toluate, and salicylate (Phe$^+$, *m*Tol$^+$, and Sal$^+$ characters, respectively).
[c] ND, Not determined.

**Transfer of Phe$^+$, *m*Tol$^+$, and Sal$^+$ characters.** We did not detect the transfer or cotransfer of the Sal$^+$ character in conjugation experiments. The *m*Tol$^+$ and Phe$^+$ characters were readily transferable from EST1001 to *P. putida* PaW340 at nearly equal frequencies ($0.8 \times 10^{-3}$ to $1.0 \times 10^{-3}$). The cotransfer of *m*Tol$^+$ and Phe$^+$ characters occurred at lower frequencies ($10^{-4}$ to $10^{-5}$) (Table 3). However, further studies revealed that these transferability results did not mean the independent transfer of genetic information of these markers. We chose the *P. putida* PaW340 transconjugants EST1010 (Phe$^-$ *m*Tol$^+$), EST1020 (Phe$^+$ *m*Tol$^+$), and EST1030 (Phe$^+$ *m*Tol$^-$) (Table 1) for further studies. Spontaneous mutants of EST1030 that were able to grow on *m*-toluate arose at a frequency of $10^{-7}$. One of them was designated EST1033. In the conjugation of Phe$^-$ *m*Tol$^+$ strain EST1010 with an isogenic recipient strain, PaW85, besides the isolation of Phe$^-$ *m*Tol$^+$ clones at a frequency of $10^{-4}$, Phe$^+$ *m*Tol$^-$ transconjugants were isolated at a frequency of $10^{-7}$ per donor cell. Phe$^+$ *m*Tol$^+$ clones also appeared at the same frequency from the cross of PaW85 with EST1030 (Phe$^+$ *m*Tol$^-$) as the donor strain.

We transformed *P. putida* Paw340 with the TOL plasmid pWWO and selected clones for the *m*Tol$^+$ phenotype. *m*Tol$^+$ clones appeared at a frequency of $10^{-5}$/μg of plasmid DNA. Despite our attempts, we did not get any Phe$^+$, *m*Tol$^+$, or Sal$^+$ transformants of PaW340 with plasmid DNA isolated from parent strain EST1001.

**Biochemical studies.** Table 4 shows the levels of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase in the parent strain *Pseudomonas* sp. strain EST1001 and in its derivatives. Enzyme assays demonstrated that in the strains studied, *m*-toluate was utilized via the catechol *meta*-cleavage pathway and phenol was degraded by *ortho*-fission of catechol. Salicylate induced both the dioxygenases in EST1001.

After exposure of parent strain EST1001 to succinate, low basal levels of both dioxygenases were detected. Our results indicate that different regulatory mechanisms exist in the parent strain and the *P. putida* PaW340 background. In contrast to transconjugants, the synthesis of catechol 1,2-dioxygenase in the parent strain was completely repressed by *m*-toluate.

In the case of EST1030 *m*Tol$^+$ mutant strain EST1033, the switchover of the phenol degradation to the *meta*-pathway took place.

Induction of phenol monooxygenase was found in the parent strain and in its transconjugants (Table 5). A significant level of phenol oxidation (20% that of the parent strain) was also detected after the exposure of *m*Tol$^+$ Phe$^-$ strain EST1010 to minimal medium containing *m*-toluate and phenol.

**Plasmid content of the strains investigated.** *m*-Toluate-cultivated cells of *Pseudomonas* sp. strain EST1001 contained several plasmid replicons (Fig. 1). Phe$^-$, Sal$^-$, and *m*Tol$^-$ derivatives of EST1001 differed from each other as well as from the parent strain in their plasmid compositions (Fig. 1A). However, the pattern of the *Hin*dIII digest of parent strain plasmid DNA was very similar to those of its derivatives EST1002, EST1003, and EST1004 (Fig. 2). These results suggest that the loss of Phe$^+$, *m*Tol$^+$, or Sal$^+$ markers of EST1001 is a result of genetic rearrangements in EST1001 plasmid DNA rather than the result of the loss of plasmid genes.

More than one plasmid replicon was also visible in PaW340 transconjugants (Fig. 1B). To investigate the extent of homology in the plasmid DNA present in strain EST1001 and its transconjugants, we carried out a series of DNA-DNA hybridization experiments.

EST1001 plasmid DNA hybridized to *Xho*I fragments of EST1020 and EST1030 plasmid DNAs at different levels (Fig. 3A and B, lanes 1 and 2). Difficulties in estimating the

TABLE 4. Specific activities of catechol dioxygenases in parent strain EST1001 and its cured derivatives and transconjugants grown on different substrates

| Substrate | Strain | Relevant phenotype | Dioxygenase activity[a] | |
|---|---|---|---|---|
| | | | C12O | C23O |
| Succinate | EST1001 | *m*Tol$^+$ Phe$^+$ Sal$^+$ | 0.01 | 0.07 |
| *m*-Toluate | EST1001 | *m*Tol$^+$ Phe$^+$ Sal$^+$ | <0.001 | 2.13 |
| | EST1002 | *m*Tol$^+$ Phe$^-$ Sal$^-$ | <0.001 | 3.37 |
| | EST1003 | *m*Tol$^+$ Phe$^+$ Sal$^-$ | <0.001 | 1.05 |
| | EST1010 | *m*Tol$^+$ Phe$^-$ Sal$^-$ | 0.09 | 2.11 |
| | EST1020 | *m*Tol$^+$ Phe$^+$ Sal$^-$ | 0.03 | 3.61 |
| | EST1033 | *m*Tol$^+$ Phe$^+$ Sal$^-$ | 0.02 | 2.46 |
| Phenol | EST1001 | *m*Tol$^+$ Phe$^+$ Sal$^+$ | 0.24 | <0.001 |
| | EST1003 | *m*Tol$^+$ Phe$^+$ Sal$^-$ | 0.32 | <0.001 |
| | EST1004 | *m*Tol$^-$ Phe$^+$ Sal$^-$ | 0.25 | <0.001 |
| | EST1020 | *m*Tol$^+$ Phe$^+$ Sal$^-$ | 0.46 | <0.001 |
| | EST1030 | *m*Tol$^-$ Phe$^+$ Sal$^-$ | 0.31 | <0.001 |
| | EST1033 | *m*Tol$^+$ Phe$^+$ Sal$^-$ | 0.01 | 1.34 |
| Salicylate | EST1001 | *m*Tol$^+$ Phe$^+$ Sal$^+$ | 0.19 | 0.80 |

[a] Expressed as micromoles of product formed per milligram of protein. Abbreviations: C12O, Catechol 1,2-dioxygenase; C23O, catechol 2,3-dioxygenase.

TABLE 5. Oxygen uptake of parent strain EST1001 and its transconjugants grown on phenol and phenol–*m*-toluate[a]

| Strain | Relevant phenotype | $O_2$ uptake by cells on the following growth substrates[b]: | |
|---|---|---|---|
| | | Phenol | Phenol–*m*-toluate |
| EST1001 | *m*Tol$^+$ Phe$^+$ Sal$^+$ | 420 | ND[c] |
| EST1010 | *m*Tol$^+$ Phe$^-$ Sal$^-$ | ND | 80 |
| EST1020 | *m*Tol$^+$ Phe$^+$ Sal$^-$ | 660 | ND |
| EST1030 | *m*Tol$^-$ Phe$^+$ Sal$^-$ | 960 | ND |

[a] In the absence of an inducer (phenol), all oxygen uptakes were <5 μl of $O_2$ consumed per h per mg (dry weight) of cells.
[b] $O_2$ uptake by whole-cell suspensions in the presence of phenol. Values are expressed as microliters of $O_2$ consumed per hour per milligram (dry weight) of cells, calculated as the difference from endogenous respiration.
[c] ND, Not determined.

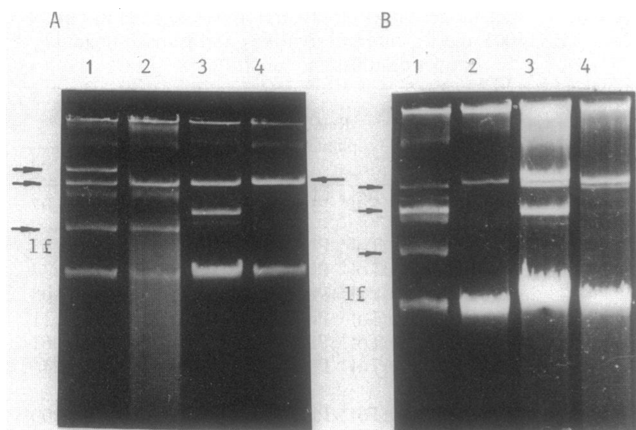


FIG. 1. Agarose gel electrophoresis of crude preparations of plasmid DNA. DNA was prepared by the method of Hansen and Olsen (20). (A) Lane 1, Parent strain EST1001; lane 2, EST1003; lane 3, EST1004; lane 4, PaW1(pWWO). (B) Lane 1, EST1001; lane 2, EST1010; lane 3, EST1020; lane 4, EST1030. The arrow on the right of panel A indicates the 117-kb TOL plasmid pWWO. The faint bands (open or nicked circular DNA and DNA-protein complexes) and the DNA band (labeled 1f; linearized fragments of chromosomal and plasmid DNAs) disappeared from CsCl-purified plasmid DNA. Arrows on the left of panel B show three ccc plasmid bands in EST1001 plasmid DNA preparations.

exact extent of homology were caused by the presence in our preparations of different quantities of each plasmid isolated from the multiplasmid strains. However, we suggest that the hybridization pattern in Fig. 3 demonstrates that the whole parent strain plasmid DNA transferred to PaW340. Hybridization of EST1001 plasmid DNA with a number of *Xho*I restriction fragments of the TOL plasmid pWWO was also seen in (Fig. 3A and B, lanes 3). Those fragments (*Xho*I-C, *Xho*I-D, *Xho*I-E, *Xho*I-I, and *Xho*I-J) are known to belong to the catabolic region of pWWO (12).

*P. putida* PaW340 is a plasmid-free strain and contains in its chromosome a 17-kb segment originating from the TOL plasmid pWWO (35). Three fragments of pWWO which were known to contain 17 kb of DNA (*Xho*I-B, *Xho*I-F, and *Xho*I-H) (35) hybridized with the PaW340 chromosome (Fig. 3C and D, lanes 2). EST1001 plasmid DNA did not hybridize with pWWO fragments *Xho*I-B, *Xho*I-F, and *Xho*I-H (Fig. 3A and B, lanes 3). We found two *Xho*I fragments from EST1030 digest which hybridized with PaW340 chromosome (Fig. 3C and D, lanes 1). The same *Xho*I fragments of pWWO which hybridized with the PaW340 chromosome appeared to be homologous with EST1030 DNA (compare lanes 2 in Fig. 3C, D, and E). Thus, from these results we concluded that the 17-kb segment of TOL DNA in the chromosome of PaW340 is translocated into the plasmid DNA of EST1030.

Several fragments of EST1020 plasmid DNA hybridized with the pWWO hot probe (Fig. 4A and B, lanes 3), showing that its plasmid DNA also contained sequences specific for pWWO.

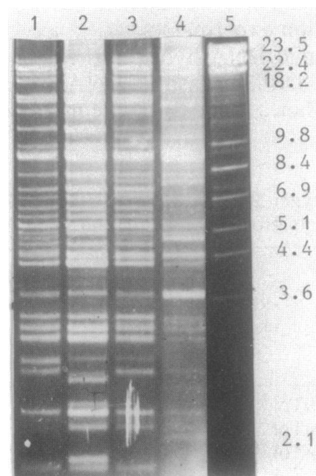


FIG. 2. Agarose gel electrophoresis of *Hin*dIII restriction endonuclease digests of crude preparations of plasmid DNA. Lane 1, EST1001; lane 2, EST1002; lane 3, EST1003; lane 4, EST1004; lane 5, PaW1(pWWO). *Hin*dIII restriction fragments of pWWO are used as size standards. The sizes (in kilobases) of pWWO fragments are those given by Downing and Broda (12).

## DISCUSSION

We studied the genetic background of phenol, *m*-toluate, and salicylate degradation in *Pseudomonas* sp. strain EST1001. The *m*-toluate-grown cells of EST1001 contained three plasmid replicons. However, none of these plasmid replicons alone was responsible for the Phe$^+$, *m*Tol$^+$, or Sal$^+$ phenotypes. There are several pieces of evidence supporting this conclusion. First, we did not get any Phe$^+$, *m*Tol$^+$, or Sal$^+$ transformants when we transformed PaW strains with plasmid DNA isolated from parent strain EST1001. Second, the restriction pattern of the plasmid DNA isolated from its eliminants EST1002, EST1003, and EST1004 was very similar to that of the parent strain, even if one of the major plasmid replicons was absent (Fig. 1A, lanes 2 and 3).

We did not detect the loss of plasmid DNA in EST1001 Phe$^-$, *m*Tol$^-$, or Sal$^-$ derivatives. Therefore, we suggest that the genetic rearrangements in EST1001 plasmid DNA are the reason why the Phe$^+$, *m*Tol$^+$, or Sal$^+$ characters can not be expressed in these segregants. Since bacteria did not lose the genetic information for the ability to degrade phenol, *m*-toluate, and salicylate, but contained it in a reorganized form, there is also the possibility that there are genetic rearrangements which lead to the reversion of wild-type bacteria.

The hybridization pattern of PaW340 multiplasmid transconjugants EST1020 and EST1033 plasmid DNA with EST1001 plasmid DNA probe (Fig. 3A and B, lanes 1 and 2) indicates that the whole parent strain plasmid DNA transferred to PaW340. While there was no independent plasmid responsible for determination of the Phe$^+$ and *m*Tol$^+$ characters in EST1001 and its transconjugants, we suppose that the utilization of phenol and *m*-toluate in these strains is specified by their whole plasmid DNA, i.e., by a multiplasmid system.

Results of our conjugation experiments indicate that *P. putida* PaW340 transconjugants always receive genetic information for strains with both phenotypes that were studied (Phe$^+$ and *m*Tol$^+$), although these genes are not expressed in all transconjugants. In several cases the conjugation of PaW340 Phe$^+$ *m*Tol$^-$ and Phe$^-$ *m*Tol$^+$ transconjugants with PaW85 resulted in the formation of transconjugants with the Phe$^+$ *m*Tol$^+$ phenotype. The switchover of phenol degradation from the *ortho*- to the *meta*-pathway in EST1030 *m*Tol$^+$ mutant strain EST1033 also showed the high flexibility of genetic material in EST1001 transconjugants.

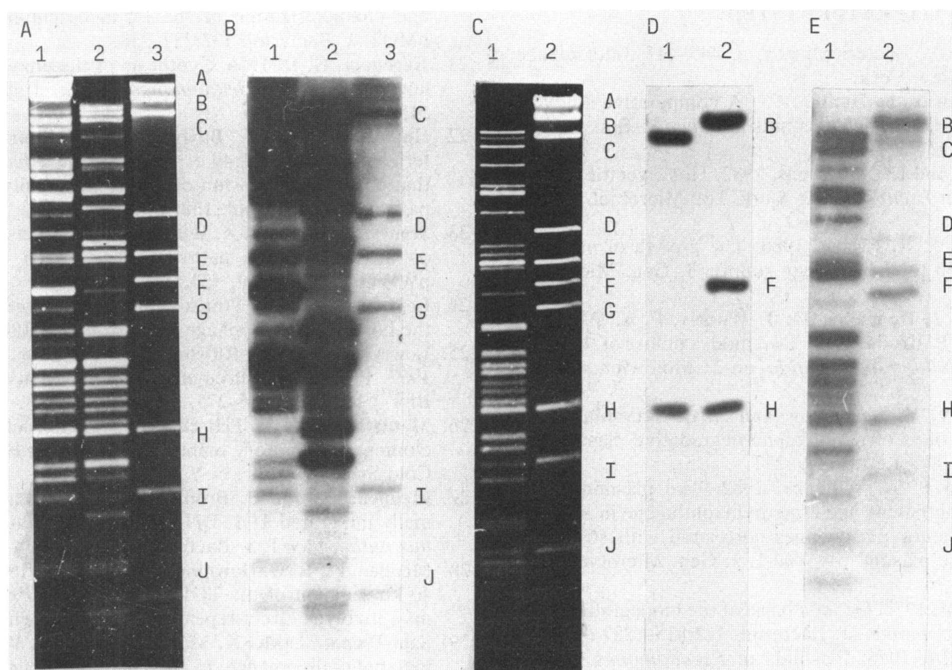


FIG. 3. (A) Agarose gel electrophoresis of *XhoI* restriction endonuclease digests of plasmid DNA. Lane 1, EST1020; lane 2, EST1030; lane 3, PaW1(pWWO). (B) Autoradiogram of the nitrocellulose-blotted DNA after hybridization with $^{32}$P-labeled EST1001 plasmid DNA. Lanes are as described above for panel A. (C) Agarose gel electrophoresis of *XhoI*-digested plasmid DNA. Lanes 1, EST1030; lanes 2, PaW1(pWWO). (D and E) Autoradiograms of the nitrocellulose-blotted DNA after hybridization with $^{32}$P-labeled PaW340 chromosomal DNA (D) and $^{32}$P-labeled plasmid DNA from EST1030 (E). Lanes are as described above for panel C. Letters A to J on the sides of the gels show pWWO *XhoI* restriction fragments, as designated by Downing and Broda (12), from top to bottom, respectively: *XhoI*-A, 51 kb; *XhoI*-B, 23 kb; *XhoI*-C, 16 kb; *XhoI*-D, 6.5 kb; *XhoI*-E, 5.3 kb; *XhoI*-F, 4.7 kb; *XhoI*-G, 4.2 kb; *XhoI*-H, 2.7 kb; *XhoI*-I, 2.3 kb; and *XhoI*-J, 1.6 kb.

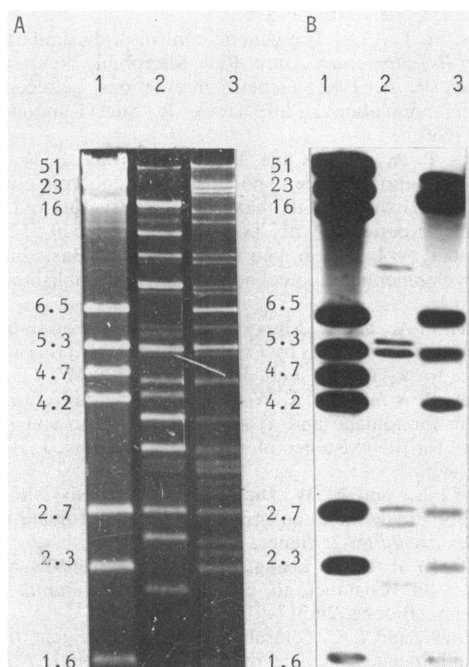


FIG. 4. (A) Agarose gel electrophoresis of *XhoI* restriction endonuclease digests of plasmid DNA. Lane 1, PaW1(pWWO); lane 2, EST1001; lane 3, EST1020. (B) Autoradiogram of the nitrocellulose-blotted DNA from the gel shown in panel A after hybridization with $^{32}$P-labeled pWWO as a probe. Numbers indicated on the side are the sizes (in kilobases) of the pWWO *XhoI* restriction fragments.

Several experiments have shown the transposition of the 17-kb TOL transposable element into other plasmids (7, 23, 32). EST1001 transconjugants in a PaW340 background carry new multiplasmid systems which differ from that of the parent strain and contain the same 17-kb sequence in their plasmids. In addition, the hybridization pattern of the EST1020 plasmid DNA *XhoI* digest with the hot pWWO probe revealed the presence of pWWO-like structure(s) in the EST1020 plasmid composition (Fig. 4A and B, compare lanes 1 and 3). Although plasmid DNA from EST1001 had definite homology with the TOL plasmid pWWO catabolic genes, the formation of pWWO-like structures from the parent strain plasmid DNA seems to be unlikely. We can only imply that pWWO-like structures are formed through cointegrate formation between EST1001 chromosomal and plasmid DNAs during the conjugal transfer and that the 17-kb transposable element, which originated from the PaW340 chromosomes, mediates the formation of these plasmids.

The molecular study of the multiplasmid systems that are present in bacteria is technically complicated. However, it cannot be avoided if one wishes to understand how biodegradative functions of soil pseudomonads have evolved and how they change in response to changes in their environment. In a forthcoming paper, we will show how a bacterial strain carrying an individual PHE plasmid can be formed from the multiplasmid parent strain.

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