JOURNAL OF BACTERIOLOGY, Apr. 1978, p. 270-277
0021-9193/78/0134-0270$02.00/0

Vol. 134, No. 1
Printed in U.S.A.

Isolation of TOL and RP4 Recombinants by Integrative Suppression

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Received for publication 6 December 1977

We obtained genetic and molecular evidence of non-thermosensitive recombinants of RP4 (Km^r Tc^r Cb^r/Ap^r) and the thermosensitive TOL plasmid. As first isolated in *Pseudomonas aeruginosa* PAO, the recombinant plasmid pTN1 specified noninducible synthesis of TOL enzymes and was transmissible to *Escherichia coli* on selection for the transfer of kanamycin resistance. The phenotypic expression of TOL genes of pTN1 in *E. coli* was low and also noninducible. A spontaneous segregant, pTN2, appearing from pTN1, conferred inducible synthesis of TOL enzymes. These plasmids carry all of the TOL determinants as evidenced by the ability of *Pseudomonas putida* carrying recombinant plasmids to grow on toluene, xylene, and *m*-toluate. In *E. coli* the expression of TOL genes with normal regulation (pTN2) appears to be extremely low without induction, and the induced expression is comparable to that with defective regulation (pTN1). The measurement of the molecular weight of pTN2 by electron microscopy gave a value of about 74×10^6.

Several degradative pathways in *Pseudomonas* are specified by genes that are not on a chromosome but are borne on separate transmissible plasmids. These include TOL, CAM, SAL, OCT, and NAH plasmids, which determine growth on toluene (or toluate), camphor, salicylate, alkanes, and naphthalene, respectively (8). These plasmids have been designated "degradative plasmids" (6).

Degradative plasmids are generally transmissible only between bacteria of the genus *Pseudomonas* (28). Among these, CAM was shown to be incompatible with the plasmids of incompatibility group P-2 (21), which was designated by Bryan et al. as one of the groups of plasmids transmissible between *Pseudomonas* species but not to *Escherichia coli* (4, 5). On the other hand, group P-1 plasmids have a remarkably wide host range (10, 26).

Recently, drug resistance determinants of *Pseudomonas*-specific plasmids from *Pseudomonas aeruginosa* to *E. coli* were mobilized by recombination with group P-1 plasmids (17, 22). Recombinants were obtained by mating *P. aeruginosa* carrying both P-1 and *Pseudomonas*-specific plasmids with *E. coli*, followed by selecting for transmission of resistance determined by *Pseudomonas*-specific plasmids. We have carried out analogous experiments to obtain recombinants of TOL and a P-1 plasmid. *P. aeruginosa* carrying both TOL and RP4, a representative plasmid of incompatibility group P-1 (11), was mated with *E. coli*, and exconjugants were selected that could grow with *m*-toluate. All such experiments failed.

In a previous paper, Nakazawa reported that the TOL plasmid carried by a strain of *P. aeruginosa* PAO exhibits thermosensitive properties of self-maintenance and inhibition of host cell growth (23). These findings prompted us to test the recombination of TOL and a non-thermosensitive plasmid based on a technique of integrative suppression. This paper describes some evidence for isolation of two types of recombinants of RP4 and TOL, which are transmissible to *E. coli*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1.

Chemicals. *p*-Methylbenzyl alcohol was obtained from Tokyo Kasei Kogyo Co. Ltd., Tokyo, and *m*-toluic acid and benzyl alcohol were obtained from Nakarai Chemical Co. Ltd., Tokyo, Japan. Disodium carbenicillin was obtained from Fujisawa Yakuhin Kogyo Co. Ltd., Osaka; kanamycin sulfate was from Meiji Seika Co. Ltd., Tokyo; and tetracycline hydrochloride was from Japan Lederle Co. Ltd., Tokyo, Japan. Other chemicals were of reagent grade and were commercially available.

Media and culture conditions. L broth containing 1% tryptone (Difco), 0.5% yeast extract (Difco), and 0.5% NaCl was adjusted to pH 7.2 and used as a

TABLE 1. *Strains and plasmids used*

Strain/Plasmid	Genotype/phenotype[a]	Reference
Strain		
P. aeruginosa PAO1	Prototroph	27
P. putida (*arvilla*) mt-2 TN2100	Prototroph (Tol$^-$)	25
E. coli 20SO	*thi lac mal xyl mtl ara rpsL*	1
Plasmid		
TOL	Tol$^+$	25
RP4	Cb^r/Ap^{r}Km^{r}Tc^r	10
pTN1	Tol^{c}Cb^r/Ap^{r}Km^{r}Tc^r	This paper
pTN2	Tol$^+$Cb^r/Ap^{r}Km^{r}Tc^r	This paper

[a] Phenotypes of plasmids: Tol$^+$, *m*-toluate utilization with inducible TOL enzymes synthesis; Tol^c, *m*-toluate utilization with noninducible TOL enzyme synthesis; Cb^r/Ap^r, carbenicillin or ampicillin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance. *E. coli* genotypes as in reference 1.

liquid medium. MacConkey agar containing galactose instead of lactose (Eiken Chemical Co. Ltd., Tokyo, Japan) and nutrient agar (Eiken) were used as solid media. Sodium benzoate (10 mM) was added to nutrient agar where indicated (BN agar). L broth was solidified with 1.5% agar (L agar) and used for the determination of minimal inhibitory concentrations. One-half-strength minimal salts medium (M9 agar) (9) was solidified with 1.5% agar and used with aromatic substrates at a concentration of 5 mM as the sole carbon source. L-Glutamate (20 mM) was added to M9 agar (G agar).

Liquid cultures were grown aerobically on a reciprocating shaker. Unless stated otherwise, incubations were carried out at 37°C for *P. aeruginosa* and *E. coli* and at 27°C for *P. putida*.

Determination of plasmid carrier fraction. Cells grown overnight in L broth in the absence or presence of 10 mM benzoate were diluted with saline, and appropriate dilutions were spread on nutrient agar. After incubation overnight at 37°C, 100 individual colonies were transferred onto nutrient agar and replicated on M9 agar containing *m*-toluate (5 mM) and on G agar containing 250 μg of kanamycin per ml.

Mating procedure. Procedures for matings were as described (25), except that incubations for matings were carried out at 37°C for *E. coli* recipients and at 27°C for *P. putida* recipients.

Selection and characterization of exconjugants. Counterselection against the donor population of *P. aeruginosa* or *P. putida* in mating with *E. coli* 20SO recipients was made by plating on M9 agar containing 0.2% trehalose and 2.5 μg of thiamine (TT agar) supplemented with 25 μg of kanamycin per ml. Cotransfer of carbenicillin and tetracycline determinants was assayed by transferring 100 individual colonies from the selective plate onto the same agar plates and replicating on TT agar containing 500 μg of carbenicillin or 10 μg of tetracycline per ml. Cotransfer of TOL determinants was assayed by streaking exconjugants on BN agar to observe the formation of yellow colonies by the accumulation of 2-hydroxymuconic semialdehyde (24).

Counterselection against the *E. coli* 20SO donor in the crosses with *P. putida* was made on M9 agar containing *m*-toluate (5 mM). Cotransfer of drug resistance determinants was assayed by transferring 100 individual colonies from the selective plate onto the same agar plates and replicating on G agar supplemented with 100 μg of kanamycin, 1,000 μg of carbenicillin, or 25 μg of tetracycline per ml. L-Glutamate did not support the growth of the donor strain.

Assays of metapyrocatechase and 2-hydroxymuconic semialdehyde hydrolyase. Procedures for preparation of crude extract, assays of metapyrocatechase (catechol 2,3-dioxygenase, EC 1.13.1.2) and 2-hydroxymuconic semialdehyde hydrolyase, as well as protein determination have been described (24, 25). One unit of enzyme activity was defined as the amount that catalyzes the formation or degradation of 1 μmol of 2-hydroxymuconic semialdehyde per min, and specific activities were defined in units of enzyme activity per milligram of protein.

Determination of the minimal inhibitory concentrations. Cells were grown overnight in L broth containing 500 μg of carbenicillin per ml. These cultures were diluted to about 10^3 cells per ml, and 1 drop from the cell suspension was placed on L agar containing various concentrations of the antibiotics being tested. The minimal inhibitory concentration is the lowest concentration of antibiotic at which no growth occurred after 24 h of incubation.

Isolation of unlabeled plasmid DNA. The procedure described by Yamamoto and Yokota (32) was used. Bacterial cells grown in L broth were harvested by centrifugation, and a clear lysate was obtained. Plasmid DNA was isolated by precipitating with polyethylene glycol, followed by cesium chloride-ethidium bromide gradient centrifugation.

Electron microscopy. The formamide technique of Davis et al. (12), modified by Ikeuchi et al. (20), was used. The spreading solution and hypophase solution used for mounting DNA contained 52 and 18% formamide, respectively. The cytochrome *c* layer with plasmid DNA was picked up on Parlodion-coated copper grids, followed by staining in uranyl acetate and rotary shadowing with platinum palladium. As an internal standard, colicin E1 DNA with a molecular weight of 4.2×10^6 was used, and the molecular weight of pTN2 was obtained from measurements of five complete molecules.

RESULTS

Isolation of temperature-resistant derivatives of *P. aeruginosa* PAO carrying TOL and RP4. Strain PAO1 of *P. aeruginosa* carrying the TOL plasmid is not able to grow with *m*-toluate above 41°C, probably because the plasmid is thermosensitive in its replication (23). Thermosensitive maintenance of TOL was not suppressed by the presence of RP4 in the same cell, which by itself replicated normally even at 43°C (Fig. 1). To obtain thermoresistant derivatives, *P. aeruginosa* PAO1 carrying both TOL and RP4 was grown in L broth at 37°C, washed once with and resuspended in M9, and spread on *m*-toluate agar. Four colonies appeared out

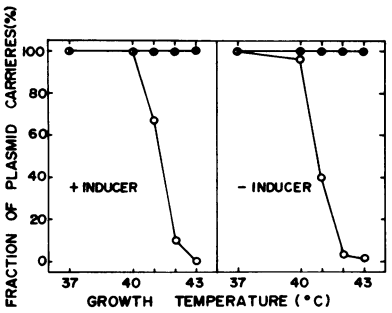

FIG. 1. *Effect of growth temperature on the stability of TOL and RP4 in P. aeruginosa PAO1 carrying TOL and RP4 in the presence (left) or absence (right) of 10 mM benzoate. An exponential culture of PAO1(TOL) (RP4) grown in L broth at 37°C was inoculated into L broth with or without benzoate at a density of 10^6/ml and incubated at various temperatures for 15 h. A sample of each culture was appropriately diluted and spread on nutrient agar to determine the fraction of TOL (○) and RP4 (●) carriers.*

of 10^8 cells on the plate after 5 days of incubation at 42°C; these were then purified on the same type of agar plate. All seemed indistinguishable, and a clone, designated TN5004, was chosen for further study.

Characterization of *P. aeruginosa* TN5004. In contrast to the parent strain carrying plasmids TOL and RP4, TN5004 did not lose the Tol$^+$ phenotype even when the cells were grown at 42°C in either the presence or the absence of an inducer (Fig. 2). An infrequent loss of the Tol$^+$ phenotype was always associated with a simultaneous loss of RP4 markers. In addition, inhibition of host cell growth by the presence of TOL (23) was observed with the parent strain, but not with TN5004 growing at 42°C in L broth (data not shown).

Besides the thermoresistant properties of TOL, TN5004 is different from the parent strain with respect to its phenotypic expression of TOL enzymes. The TOL plasmid specifies enzymes of the *meta* pathway, which convert benzoate, *m*-toluate, and *p*-toluate to pyruvate and either acetaldehyde or propionaldehyde (29). Table 2 shows that the two coordinately controlled enzymes of the *meta* pathway, metapyrocatechase and 2-hydroxymuconic semialdehyde hydrolyase (16), are induced by benzoate in the parent strain, whereas those in TN5004 are not induced at either 37 or 42°C. The levels of both enzymes of TN5004 are about twice as much as the noninduced levels of the parent. All of the derivatives isolated so far as thermoresistant strains able to utilize *m*-toluate had noninducible synthesis of the TOL enzymes.

Transfer of TOL genes from *P. aeruginosa* to *E. coli*. The TOL plasmid is transmissible only between strains of the fluorescent group of *Pseudomonas* (23), although some TOL plasmids are isolated in nonfluorescent *Pseudomonas* sp. (30). Several attempts to transfer the plasmid to *E. coli* were unsuccessful. To determine whether the thermoresistance of TOL in TN5004 is due to the integration of TOL genes into the RP4 replicon, their transmissibility to *E. coli* was tested. Thus, strain TN5004 was mated with *E. coli* 20SO, and exconjugants were selected on TT agar containing kanamycin. Exconjugants obtained at a frequency of 10^{-5} per donor cell also showed carbenicillin and tetracycline resistance, but they did not show ability to grow with *m*-toluate or benzoate. It was possible, however, that the expression of TOL genes in *E. coli* might not have been sufficient to allow cell growth with these compounds as a sole source of carbon.

To test this possibility, 20 exconjugants puri-

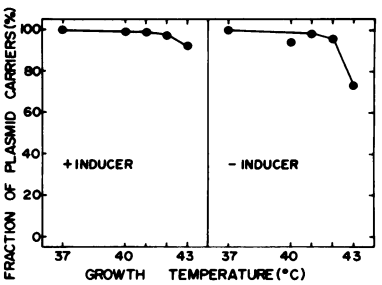

FIG. 2. *Effect of growth temperature on the stability of TOL and RP4 in TN5004 in the presence (left) or absence (right) of benzoate. Culture conditions for growth and procedures for determining TOL and RP4 carriers were identical to those described for PAO1 in Fig. 1. Closed circles indicate carriers of both plasmids.*

TABLE 2. *TOL enzyme levels in strains PAO1(TOL)(RP4) and TN5004*[a]

Strain	Addition of inducer	Growth temp (°C)	Sp act (U/mg of protein)[b]	
			Metapyrocatechase	2-Hydroxymuconic semialdehyde hydrolyase
PAO1(TOL)	−	37	0.80	0.019
(RP4)	+	37	9.27	0.147
TN5004	−	37	1.88	0.027
	+	37	1.68	0.029
	−	42	1.30	0.031
	+	42	1.65	0.042

[a] Cells were grown overnight in L broth in the absence or presence of 10 mM benzoate as an inducer.

[b] Enzyme activities were determined in crude extracts as described in the text.

fied on galactose-MacConkey agar were streaked on BN agar to see if the yellow intermediate, 2-hydroxymuconic semialdehyde, was formed. All exconjugants tested formed yellowish-brown colonies, suggesting the accumulation of 2-hydroxymuconic semialdehyde from benzoate via catechol by the action of metapyrocatechase (24). Since these properties, as well as drug resistance properties, of exconjugants were stable, one of them, designated 20SORT1, was used for the determination of levels of *meta*-pathway enzymes. Table 3 shows the presence of both metapyrocatechase and 2-hydroxymuconic semialdehyde hydrolyase activities in the crude extract of 20SORT1. The levels were not affected by inducer and were 70 times lower than those in TN5004. These enzyme activities were not detected in the crude extract of *E. coli* 20SO. Since accumulation of 2-hydroxymuconic semialdehyde from benzoate requires benzoate oxygenase complex in addition to metapyrocatechase, the above findings indicate that at least three enzymes determined by the TOL plasmid are synthesized by *E. coli* 20SORT1. The TOL genes in *E. coli* were further transmissible to *P. putida* together with RP4 markers, as shown below. Thus, the plasmid carried by 20SORT1 was designated pTN1, a recombinant plasmid of RP4 and TOL coding for noninducible synthesis of TOL enzymes.

TABLE 3. *TOL enzymes in curde extracts of E. coli exconjugant, 20SORT1*[a]

Strain	Addition of inducer (mM)	Sp act (U/mg of protein)	
		Metapyrocatechase	2-Hydroxymuconic semialdehyde hydrolyase
20SORT1	None	0.0267	0.0003
	Benzoate (1)	0.0250	0.0003
	Benzoate (5)	0.0184	0.0003
20SO	None	<0.0001	<0.0001
	Benzoate (5)	<0.0001	<0.0001

[a] Cells were grown overnight in L broth at 37°C with shaking in the absence or presence of benzoate.

TABLE 4. *TOL enzyme levels in crude extracts of P. putida (arvilla) mt-2 strains*[a]

Strain	Addition of inducer	Sp act (U/mg of protein)	
		Metapyrocatechase	2-Hydroxymuconic semialdehyde hydrolyase
TN2100RT1	−	0.186	0.0039
	+	0.189	0.0036
TN2100RT2	−	0.220	0.0036
	+	3.31	0.0394
TN100(TOL) (RP4)	−	0.046	0.0021
	+	1.62	0.0272

[a] Cells were grown overnight in L broth in the absence or presence of 10 mM benzoate as an inducer.

Transfer of pTN1 from *E. coli* to *P. putida* and isolation of a recombinant plasmid providing inducible TOL enzymes. To see if the entire TOL determinant was incorporated into plasmid pTN1, *E. coli* 20SORT1 was mated with a TOL$^-$ derivative of *P. putida* (*arvilla*) mt-2, the original host of the TOL plasmid, and exconjugants were selected on *m*-toluate. After incubation for 3 days, exconjugants appeared at a frequency of 10^{-4} per donor cell and mostly formed large colonies on the selective plate. A few exconjugants forming very small colonies were also obtained. Resistance markers of RP4 were carried by all exconjugants examined. The growth properties of large-colony formers and small-colony formers did not change during purification on *m*-toluate. Thus, two exconjugants, TN2100RT1 and TN2100RT2, representing small- and large-colony formers, respectively, were chosen for the determination of TOL enzyme levels. Table 4 shows that strain TN2100RT1 produces TOL enzymes noninducibly at about 10 times the level found in *E. coli* 20SORT1. On the other hand, TN2100RT2 produces TOL enzymes inducibly, which is similar to TN2100 carrying separate plasmids.

These findings suggest that pTN1, first isolated in *P. aeruginosa* PAO, has genes responsible for the complete degradation of *m*-toluate. The conversion of the TOL determinants from a noninducible to an inducible state might occur spontaneously during or after the transfer of pTN1 from *E. coli* to *P. putida*, and exconjugants producing TOL enzymes inducibly at high levels were effectively selected on *m*-toluate. Such a conversion also occurred at a frequency of 10^{-4} when TN2100RT1 cells were spread on *m*-toluate agar and incubated at 27°C for 5 days.

In addition to determinants of the enzymes responsible for the degradation of benzoate, *m*-toluate, and *p*-toluate, the TOL plasmid also carries the genes for the enzymes governing catabolism of toluene, *m*-xylene, and *p*-xylene, which are metabolized through the corresponding alcohols and aldehydes to benzoate and *m*- and *p*-toluate, respectively (31). These genes are also carried by TN2100RT2 and possibly TN2100RT1, because these strains could grow with toluene, *m*-xylene, *p*-xylene, benzyl alcohol, and *p*-methylbenzyl alcohol as a sole source of carbon (Table 5).

Although TN2100RT2 is similar to TN2100, carrying determinants of the inducible expression of TOL enzymes, it could donate TOL de-

terminants to *E. coli* together with RP4 markers, as shown below. Therefore, TOL genes of TN2100RT2 may harbor a recombinant plasmid between TOL and RP4; this plasmid was designated pTN2, a recombinant plasmid providing inducible synthesis of TOL enzymes.

Phenotypic properties of the recombinant plasmid in *E. coli*. TN2100RT2 was mated with *E. coli* 20SO, and exconjugants were selected for the transfer of kanamycin resistance. *E. coli* exconjugants thus obtained carried RP4 antibiotic resistance markers and formed yellow colonies on BN agar but were unable to grow with *m*-toluate or benzoate. Table 6 summarizes some phenotypic properties of pTN1 and pTN2 in *E. coli*. Specific activities of metapyrocatechase in the crude extract of strain 20SO carrying pTN2 is extremely low without induction, and its induced level is comparable to that of *E. coli* 20SO carrying pTN1. Since minimal inhibitory concentrations of antibiotics determined in the absence of inducer are essentially the same for strains carrying pTN1, pTN2, or RP4, the plasmid copy numbers may not be different from each other in the host bacteria.

Measurement of the molecular weight of pTN2. A recombinant plasmid, pTN2, in *E. coli* 20SO was labeled with [^{3}H]thymine and separated by cesium chloride-ethidium bromide gradient centrifugation. Since the plasmid behaved on neutral sucrose gradient centrifugation as unimolecular species of higher molecular weight than RP4 (data not shown), its molecular weight was determined by electron microscopy. Unlabeled pTN2 was isolated by cesium chloride-ethidium bromide gradient centrifugation. Figure 3 shows a molecule of pTN2 in open circular form. The average molecular weight of 73.7 ± 2.5 (n = 5) × 10^6 was calculated from length determinations by electron microscopy. The molecular weight of RP4 determined as a control in these experiments was 37.3 ± 1.1 (n = 5) × 10^6, which is very close to a reported value of 36 × 10^6 (22). TOL is 75 to 78 megadaltons in mass (8, 13), and therefore pTN2 is not simply the fusion product of TOL and RP4.

DISCUSSION

In a previous paper, Nakazawa showed the thermosensitive properties of the TOL plasmid in *P. aeruginosa* PAO (23). Based on these observations, we attempted to isolate a recombinant plasmid between TOL and RP4 by the technique of integrative suppression, as suggested by Chakrabarty (7). The present work suggests that a derivative selected as a suppressed mutant of thermosensitive TOL maintenance from *P. aeruginosa* PAO carrying both TOL and RP4 contains a recombinant plasmid of TOL and RP4.

Benedik et al. (3) have reported that RP1, indistinguishable from RP4 (19, 22), transposes the beta-lactamase gene into the SAL plasmid and also into the camphor genes of the CAM-OCT plasmid. Recombination between RP4 and TOL described here is different from the transposition of RP4 genes into degradative plasmids, since the recombinant plasmids possess not only the carbenicillin resistance gene, but also the kanamycin and tetracycline resistance genes in addition to TOL determinants. Compatibility tests of these plasmids are currently under way.

TABLE 5. *Growth properties of P. putida (arvilla) mt-2 exconjugants*

Strain	Growth property[a]				
	Toluene	*m*-Xylene	*p*-Xylene	*p*-Methylbenzyl alcohol	Benzyl alcohol
TN2100RT1	+	+	−	+	+
TN2100RT2	+	+	+	+	+
TN2100(TOL)(RP4)	+	+	+	+	+

[a] Cells were grown at 27°C for 5 days on M9 agar containing 5 mM aromatic substrate. +, Growth; −, no growth.

TABLE 6. *Phenotypic expression of recombinant plasmids in E. coli 20SO*

Plasmid	Sp act of metapyrocatechase (U/mg of protein)		Minimal inhibitory concn (μg/ml)		
	−Inducer	+Inducer[a]	Kanamycin	Tetracycline	Carbenicillin
pTN1	0.0267	0.0184	250	50	>20,000
pTN2	0.0026	0.0248	250	50	>20,000
RP4	ND[b]	ND	250	50	>20,000

[a] Benzoate (5 mM) was added to the growth medium.
[b] ND, Not determined.

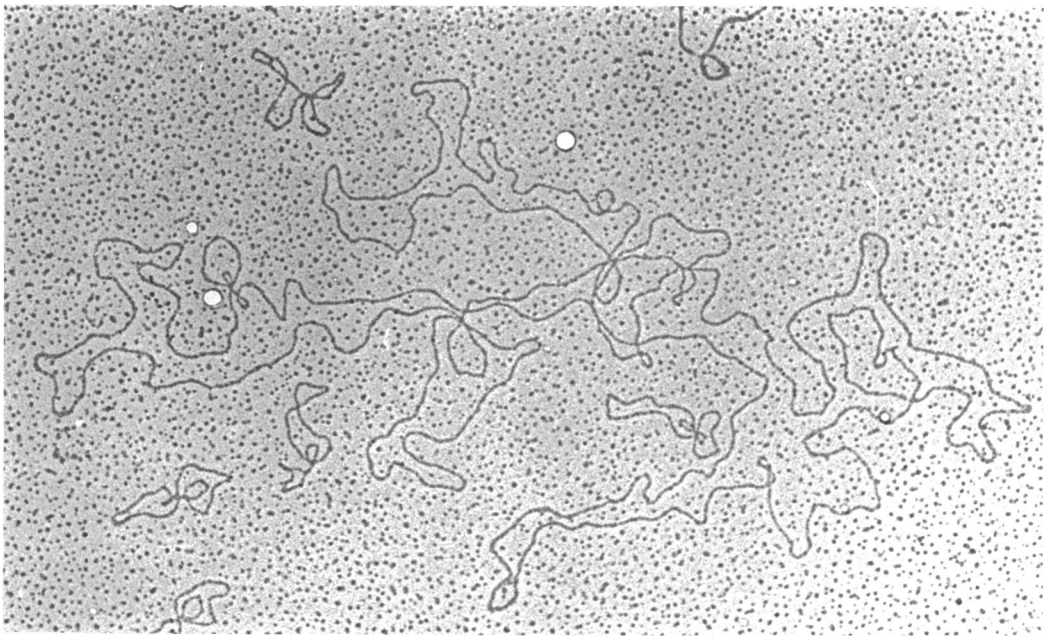

FIG. 3. *Electron micrograph of an open circular molecule of pTN2 plasmid DNA purified from E. coli 20SO (pTN2). Small twisted molecules are colicin E1 DNA added as an internal standard.*

Although the mechanism of recombinant formation is not clear, it may be worthwhile to point out that the recombinant plasmid first isolated in *P. aeruginosa* TN5004 provides synthesis of the TOL enzymes noninducibly. Thermoresistant derivatives isolated so far showed properties similar to those of TN5004, suggesting that they also contain plasmid pTN1. A spontaneous change or mutation from pTN1 to plasmid pTN2, conferring inducible synthesis of TOL enzymes, indicates that a regulatory gene is not deleted in pTN1. It is not known whether pTN2-like plasmids either never formed directly in *P. aeruginosa* PAO or were formed but were not isolated under the selective conditions.

Recent work with P-1 plasmids showed that they can incorporate resistance markers from *Pseudomonas*-specific plasmids and transmit them into *E. coli* (17, 22). In these cases, the resistance-determining genes of *Pseudomonas*-specific plasmids function in *E. coli* to allow isolation of recombinant plasmids by selecting for the transfer of a resistance gene of the plasmids from *Pseudomonas* to *E. coli*. Analogous experiments attempting to isolate recombinant plasmids of TOL and RP4 by selecting *E. coli* exconjugants able to grow with *m*-toluate were not successful, because the expression of the TOL genes in *E. coli* is not sufficient to support the growth. The enzyme level of TOL conferred by pTN1 in *E. coli* (Table 3) is 70 times lower than that in the original host (Table 2), or 7 times lower than that in *P. putida* (Table 4). Since the growth of *P. putida* carrying pTN1 is very slow with *m*-toluate, forming only tiny colonies after 4 days of incubation, it may not be surprising that *E. coli* 20SO carrying pTN1 is not able to grow with *m*-toluate.

Such an impediment to the expression of *Pseudomonas* genes in *E. coli* has recently been reported by Hedges et al. (18), who studied the incorporation of the chromosomal *trpAB* genes from *P. aeruginosa* into plasmid R64.44 and the expression of these genes after transfer to *E. coli*. An impediment to the expression of TOL genes with normal regulation (pTN2) appears to be more severe than that with defective regulation (pTN1). Thus, the induced level of metapyrocatechase conferred by pTN2 in *E. coli* (Table 6) is more than 100 times lower than that in *P. putida* (Table 4). Although the gene copy number of the recombinant plasmids in these bacteria is not known, such a great difference between the enzyme level in *Escherichia* and that in *Pseudomonas* cannot be explained only by a difference in the plasmid copy number.

The phenotypic properties of the recombinant plasmids in *E. coli* (Table 6) shows that the metapyrocatechase level provided by pTN1 is essentially the same as its induced level provided by pTN2. Furthermore, the results of minimal inhibitory concentration determinations of an-

tibiotics suggest that the copy number of the recombinant plasmids is probably the same as that of RP4, i.e., one to three copies per chromosome (15, 22), if RP4 genes are equally expressed. The simplest explanation for these findings may be that pTN2 produces a repressor controlling the TOL operon negatively, and pTN1 is a mutant that has no functional repressor.

The phenotypic expression of TOL genes in *Pseudomonas* is apparently different from that in *E. coli*. The TOL enzyme level provided by pTN1 is essentially the same as its noninduced level provided by pTN2 in *P. putida* (Table 4). To explain this, a positive control model for the arabinose operon rather than the negative control model described for the lactose operon (14) seems to be suitable.

In addition to the genes for the *meta*-pathway enzymes responsible for the degradation of benzoate, *m*-toluate, and *p*-toluate, the TOL plasmid also carries genes for the catabolism of toluene, *m*-xylene, and *p*-xylene to benzoate and *m*- and *p*-toluate, respectively. These genes probably form a regulatory unit independent from those for the *meta*-pathway enzymes (31). pTN1 and pTN2 seem to carry both regulatory units, but it is not clear whether the regulation of enzymes other than those of the *meta* pathway is also defective in pTN1.

Recently, Bayley et al. (2) reported a spontaneous deletion of a specific region of about 27 megadaltons from the TOL plasmid. Since the shortened plasmid lost the Tol^+ phenotype, the presence of "hotspots" was suggested for the excision of a segment that codes for at least part of the TOL pathway. It is possible that these hotspots are responsible for the recombination of TOL and RP4. The molecular basis for recombinant formation is currently under investigation.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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