Physical Mapping of TOL Plasmids pWWO and pND2 and Various R Plasmid-TOL Derivatives from *Pseudomonas* spp.

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Analysis of several independently isolated R plasmid-TOL hybrids revealed a wide variation in the amount of TOL DNA they contain. If the formation of the various R plasmid-TOL hybrids involves transposition (which has yet to be rigorously assessed), such transposition does not involve a unique segment of TOL DNA.

The TOL plasmid of *Pseudomonas putida* mt-2 confers on its host the ability to utilize toluene and several toluene derivatives (11, 16, 17). A physical map of the archetypal TOL plasmid, pWWO, has been published (3), and several functions have been mapped on the restriction enzyme map of pWWO (6) and of an RP4-TOL derivative (13). Early in the analysis of the TOL (pWWO) plasmid, it was shown that recombinants between R plasmids and pWWO could be isolated (2, 14, 15). This has been explained as "transposition" of segments of the pWWO plasmid into the R plasmids studied (2, 10). However, it has not been rigorously demonstrated whether the recombinant formation fulfills the criteria of true transposition.

This paper describes the isolation of a new R plasmid-TOL recombinant and the physical analysis of this and several other R plasmid-TOL recombinants. The new R plasmid-TOL hybrid is a recombinant between the Inc-W group plasmid Sa (9, 14a) and the Tol plasmid pND2 (15). The plasmid pND2 was isolated by White and Dunn (15) after a plate mating between *P. putida* PAri-6 (TOL) and *Pseudomonas aeruginosa* PAO3 (*trp*⁻). The ability of PAO3 exconjugants to grow on *m*-toluate was rapidly lost under nonselective conditions (growth in Luria broth); however, a stable TOL⁺ variant, strain PAO3 (pND2), was isolated (15). We have compared pND2 with pWWO by using the enzymes *Eco*RI, *Hind*III, and *Xho*I and have seen no differences between them. No gross structural changes, therefore, are associated with the stability of the TOL plasmid pND2 in

P. aeruginosa PAO. The ability to degrade *m*-toluate was readily transferred from *P. aeruginosa* PAO3 pND2 to *P. putida* AC34 (2×10^{-2} transconjugants per donor cell), whereas transfer to other strains of *P. aeruginosa* PAO was not observed ($<10^{-6}$ transconjugants per donor cell). This suggests that the stability of pND2 in *P. aeruginosa* PAO is due to a host mutation(s). Curing and reinfection experiments would be necessary to test this hypothesis.

An earlier report (2) also describes the isolation of PAO (Tol⁺) exconjugants. In this case the strains were unable to transfer the TOL plasmid (termed TOL*) to either *P. aeruginosa* or *P. putida* recipients. TOL* is also considerably smaller (28 megadaltons) than the original TOL plasmid (2). These properties thus differ from those of pND2; TOL* and pND2 therefore may reflect different mechanisms by which TOL is established in *P. aeruginosa* PAO.

To allow more detailed analyses of pWWO, pND2, and the various R plasmid-TOL derivatives, we have constructed an *Eco*RI cleavage map of pWWO. *Eco*RI digests of pWWO DNA give 29 fragments (5), compared with the *Hind*III (19 fragments) and *Xho*I (10 fragments) digests (3). The *Eco*RI map was deduced from a series of experiments with double (*Hind*III, *Eco*RI and *Xho*I, *Eco*RI) digests of individually cloned *Hind*III fragments of pWWO (unpublished data). Bacteriophage lambda DNA digested with *Hind*III and pWWO DNA digested with *Hind*III and *Xho*I (3) were used as DNA size markers.

The new R plasmid-TOL recombinant was isolated as detailed below. A loopful of PAO3 (pND2, Sa) cells that had been grown overnight in nutrient broth at 42°C was used in a plate mating with *P. aeruginosa* PAO2 (*ser*⁻). Co-transfer of a drug resistance marker (gentamicin

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resistance) and the Tol⁺ phenotype were selected for by plating on minimal medium supplemented with serine (20 $\mu\text{g ml}^{-1}$), *m*-toluate (10 mM), and gentamicin (20 $\mu\text{g ml}^{-1}$). From the colonies that appeared, a clone containing an Sa-TOL hybrid plasmid (pMT101) was obtained.

The R plasmid-TOL recombinants studied are shown in Fig. 1. Purified plasmid DNA preparations from *P. putida* PU21 (pED3304), an RP4-TOL recombinant (10), *P. aeruginosa* PAO3 (pND3), an R91-TOL recombinant (15), and *P. aeruginosa* PAO2 (pMT101), an Sa-TOL recombinant, were restricted with *Eco*RI, *Hind*III, and *Xho*I and compared with similar digests of pWWO or pND2. The plasmid pED3304 has a continuous 69-kilobase region of TOL inserted into a single site in RP4 within the tetracycline resistance gene. The *Hind*III digests show that fragments HB, HC, HS, HR, HJ, and HG are

missing or incomplete. The endpoints of TOL DNA could now be more accurately mapped with reference to the *Eco*RI digests; one endpoint within HG occurs within the 1.8-kilobase region between the end of the *Eco*RI EW fragment (intact in pED3304) and the end of HG. The other endpoint is within the small *Hind*III fragment, HS.

The R91-TOL hybrid, pND3, contains the largest continuous segment of TOL DNA (approximately 104 kilobases); all the TOL *Hind*III fragments except HC, HG, HJ, and HR are present. The endpoint within HG appears to be similar to that of pED3304; the other endpoint, within HC, is in *Eco*RI fragment ER or EJ.

Digests with *Hind*III show that the Sa-TOL hybrid, pMT101, and the RP4-TOL hybrid, pTN2, contain the smallest segment of TOL DNA among the plasmids studied (approximate-

FIG. 1. Restriction enzyme cleavage map of the TOL plasmid pWWO (and pND2). Fragments produced by each enzyme (*Eco*RI, *Hind*III, and *Xho*I) are labeled alphabetically in order of decreasing size. The extent of TOL DNA present in various R plasmid-TOL derivatives is indicated by solid continuous lines. One end of pND3 is dotted to indicate that the endpoint of TOL DNA is in *Eco*RI fragment EJ or ER. The numbers on the inner ring represent kilobases (kb).

ly 56 kilobases). The *Hind*III fragments HB, HC, HR, HJ, HG, HE, HS, HI, and HK are missing or incomplete. One endpoint is within the TOL *Eco*RI EI fragment (and HE), and the other endpoint is within HK (and *Eco*RI EX). Interestingly, pMT101 lacks 5.4 kilobases of Sa and consequently chloramphenicol resistance. However, this type of deletion event has been reported before for Sa (8) and thus may not be related to the formation of the Sa-TOL hybrid.

Data from several other independently isolated RP4-TOL plasmids have recently been summarized (12). Further analysis of *Eco*RI digests of these hybrids (pED3300, pED3301, pED3302, and pED3303) allowed accurate determination of the amounts of TOL DNA that they carried. Also included in this examination was the previously characterized RP4-TOL plasmid pTN2 (13). The results presented in Fig. 1 allow a comparison of all the plasmids analyzed.

Several conclusions can be drawn from these studies. First, R plasmids of various incompatibility groups can be used to form hybrid plasmids carrying TOL catabolic genes. Second, with the exception of the Sa derivative studied, all the R plasmid DNA is present in the hybrid molecules. Third, all the R plasmid-TOL hybrids examined include and extend at both ends beyond a 40-kilobase region (between *Hind*III HD and HF) previously known to contain the TOL catabolic genes (1). This region is bounded by direct repeats (12) and is lost after growth of PAW1 on benzoate (1, 4). Similarly, pND2 and various of the R plasmid-TOL hybrids lose this 40-kilobase region when grown on benzoate (unpublished data).

Finally, the amount of TOL DNA incorporated in R plasmids varied from 56 to 104 kilobases. It is not clear at this stage whether this variation in the amount of TOL DNA reflects differences in the method of selection and isolation of R plasmid-TOL hybrids. In the case of pMT101 (this study) and pTN2 (13, 14), integrative suppression by selecting for the Tol⁺ phenotype and drug resistance at 42°C in *P. aeruginosa* PAO led to the integration of the same 56-kilobase region of TOL into Sa and RP4, respectively. However, a similar selection for the stabilization of TOL at 37°C in *P. aeruginosa* PAO R91-containing cells produced a hybrid plasmid (pND3) containing 104 kilobases of TOL DNA. By using the method of cotransfer of the Tol⁺ phenotype and particular drug resistances, several of the RP4-TOL derivatives (pED3304 [10], pED3300 [7], and pED3301 [P. Williams, personal communication]) were independently isolated. In all these cases, a 69-kilobase region of TOL has become incorporated into the RP4 plasmid. Two groups (2, 10) have suggested that transposition of the Tol⁺

phenotype may be responsible for the formation of RP4-TOL hybrids; however, it is clear from our data that such transposition could not involve a unique segment of TOL DNA. We are currently investigating the junction restriction fragments of the R plasmid-TOLs DNA in order to understand the formation of these hybrids.

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