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# SAL-TOL In Vivo Recombinant Plasmid pKF439

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**SAL-TOL in vivo recombinant plasmid pKF439 was characterized in a strain from a mixed culture of bacteria harboring various degradative plasmids. Analysis of the gene organization of pKF439 revealed that the 57-kilobase TOL fragment, including the 40-kilobase TOL metabolic region, was inserted into the complete SAL replicon at the position of *Sma*I-C within *Xho*I-B of SAL. The molecular size of pKF439 was calculated to be 138 kilobases. pKF439 could be transferred to *Pseudomonas putida* and *Pseudomonas aeruginosa* at high frequency, and the transconjugants gained the ability to grow with *m*-xylene, *m*-toluate, and salicylate as the sole carbon source.**

Among the degradative plasmids, TOL(pWW0) and SAL have been characterized by a number of workers. TOL confers the ability to utilize toluene and several toluene derivatives on *Pseudomonas putida*. It has been shown that some portions of the TOL plasmid pWW0 could be fused to some R factors, such as RP4 (12, 18), R91 (20), R2 (14), pMG18 (14), and Sa (16). Jeenes and Williams (14) and Chatterjee and Chakrabarty (5) also demonstrated that the 56- to 57-kilobase (kb) portion of TOL was integrated into the chromosomal DNA in a *Pseudomonas* sp. and the cells gained TOL functions. The SAL plasmid, which confers salicylate metabolism, was first found by Chakrabarty (3). A close relationship between SAL and NAH was postulated on the basis of metabolic and genetic data (11, 25, 26). TOL(pWW0) and SAL belong to the same P9 incompatibility group (2) and therefore cannot coexist stably in descendants of the same host cells.

Using the method of Kellogg et al. (15), we have isolated *P. putida* KF439, which is able to grow on *m*-xylene and also on salicylate. The same strain harbored a large conjugative 138-kb SAL-TOL recombinant plasmid, pKF439, whose characteristics are described here.

In the course of isolating bacteria that degrade polychlorinated biphenyls in mixed chemostat cultures of strains carrying various degradative plasmids (Table 1), we obtained strain KF439, which could grow well not only on *m*-xylene and *m*-toluate, but also on salicylate. The growth properties of strain KF439 are presented in Table 2 and compared with those of TOL(pWW0), SAL, and NAH7 plasmids in *P. putida*. A single large (138-kb) plasmid (pKF439) was isolated from strain KF439.

To examine whether pKF439 encoded the Xyl$^+$ Tol$^+$ Sal$^+$ characters, mating was conducted on a membrane filter between strain KF439 (Xyl$^+$ Tol$^+$ Sal$^+$ Str$^s$) and *P. aeruginosa* AC590 (Xyl$^-$ Tol$^-$Sal$^-$ Trp$^-$ Str$^r$). All transconjugants grown on the selection media (*m*-toluate or salicylate, both with streptomycin [300 μg/ml]) had the Xyl$^+$ Tol$^+$ Sal$^+$Trp$^-$ Str$^r$ phenotype. The Xyl$^+$ Tol$^+$Sal$^+$ *P. aeruginosa* AC590 transconjugants retained a single large 138-kb plasmid identical to pKF439. Since it was found that pKF439 confers the Xyl$^+$ Tol$^+$ Sal$^+$ phenotype, strain AC590(pKF439) was then used as a donor for further mating experiments. High conjugal transfer of pKF439 was observed for recipient strains *P. putida* AC10 ($3 \times 10^{-3}$ per donor cell), KF111 ($10^{-2}$), and KT2440 ($4 \times 10^{-3}$) and *P. aeruginosa* PAO1161 ($5 \times 10^{-3}$).

Endonuclease digestion with *Eco*RI, *Bam*HI, *Hin*dIII, *Sma*I, and *Xho*I revealed that pKF439 possessed a number of common DNA fragments from the SAL and TOL(pWW0) plasmids. These pKF439 fragments were confirmed to have homology with SAL and TOL(pWW0) by Southern blot hybridization analysis (19). We digested pKF439 with *Sma*I and *Xho*I and performed blot hybridizations with $^{32}$P-labeled pKF439 as a probe (Fig. 1). Among 15 fragments of pKF439 generated by *Xho*I digestion, fragments A, D, F, L, and O were derived from SAL, fragments C, E, G, H, I, K, M, and N were derived from TOL, and fragments B and J were junction DNAs which came from both SAL and TOL. These results clearly demonstrated that a 57-kb TOL segment had inserted into the complete SAL replicon at the position of

TABLE 1. Bacterial strains used

| Strain | Plasmid | Relevant genotype or phenotype[a] | Source or reference |
|---|---|---|---|
| *P. putida* | | | |
| KF439 | pKF439 | Xyl$^+$ Tol$^+$ Sal$^+$ | This work |
| AC10 | | *met* | A. M. Chakrabarty |
| KF111 | | | This work |
| KT2440 | | | K. N. Timmis |
| AC545 | SAL | *met* Sal$^+$ | A. M. Chakrabarty |
| AC137 | TOL(pWW0) | *met* Xyl$^+$ Tol$^+$ | A. M. Chakrabarty |
| PpG1064 | NAH7 | *trp* Nah$^+$ | I. C. Gunsalus |
| AC866 | pAC27 | 3Cba$^+$ 4Cba$^+$ | 6, 7 |
| *P. aeruginosa* | | | |
| AC590 | | *trp* Str$^r$ | A. M. Chakrabarty |
| PAO1161 | | *leu hsdR hsdM* | B. W. Holloway |
| *Acinetobacter* sp. | | | |
| KF424 | pKF1 | BP$^+$ | 8 |

[a] Phenotype designations denote the ability to grow on *m*-xylene (Xyl$^+$), *m*-toluate (Tol$^+$), salicylate (Sal$^+$), naphthalene (Nah$^+$), 3-chlorobenzoate (3Cba$^+$), 4-chlorobenzoate (4Cba$^+$), and biphenyl (BP$^+$) as sole carbon sources. Str$^r$, Resistance to streptomycin; *met*, *trp*, and *leu*, requirement for methionine, tryptophan, and *leucine*, respectively.

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TABLE 2. Comparison of growth properties[a]

| Substrate | KF439 (pKF439) | AC137 (pWW0) | AC545 (SAL) | PpG1064 (NAH7) |
|---|---|---|---|---|
| *m*-Xylene | + | + | − | − |
| *m*-Tolualdehyde | + | + | + | + |
| *m*-Methylbenzyl alcohol | + | + | − | − |
| *m*-Toluate | + | + | + | + |
| 1,2,4-Trimethylbenzene | + | + | − | − |
| Naphthalene | − | − | − | + |
| Salicylate | + | − | + | + |

[a] *m*-Xylen, *m*-tolualdehyde, *m*-benzylalcohol, and 1,2,4-trimethylbenzene were supplied as a vapor on the lid of a petri dish which was sealed with vinyl tape.

*Sma*I-C within fragment *Xho*I-B, which occurs within the 6.1-kb *Hin*dIII fragment G of TOL. The other end point within *Xho*I-B occurred within the 5.0-kb *Hin*dIII fragment H of TOL (Fig. 2). The molecular size of pKF439 was calculated to be 138 kb. Two DNA segments of *Xho*I-digested pKF439 (fragments D and M) were hybridized with the $^{32}$P-labeled *xylE* gene of TOL, which encodes catechol 2,3-oxygenase (data not shown), indicating that *xylE* and *nahH* (the catechol 2,3-oxygenase gene of SAL and NAH plasmids [25]) had extensive homology.

Since it is known that TOL(pWW0) is prone to deletion or cure by growth on benzoate (1), pKF439-harboring strains were grown on benzoate. Unlike pWW0, pKF439 was rather stably maintained in host *P. putida* and *P. aeruginosa* strains for about 40 generations. The curing frequency of pKF439 was 10% in strain KT2440(pKF439), 4% in strain AC590(pKF439), and 2% in strain KF111(pKF439). No plasmid loss was observed in strains KF439(pKF439), AC10(pKF439, or PAO1161(pKF439). Furthermore, 2 to 12% of cells from pKF439-carrying strains AC10, KF111, KT2440, and AC590 showed the Xyl$^-$ Tol$^-$ Sal$^+$ phenotype. The plasmid (pKF439Δ) from those cells was found to carry a deletion in the region of the inserted TOL DNA (data not shown). The deleted TOL DNA was a 40-kb region which contained essential TOL catabolic determinants (Fig. 2). The same deletion event was observed previously in pWW0 by Meulien et al. and others (13, 14, 17).

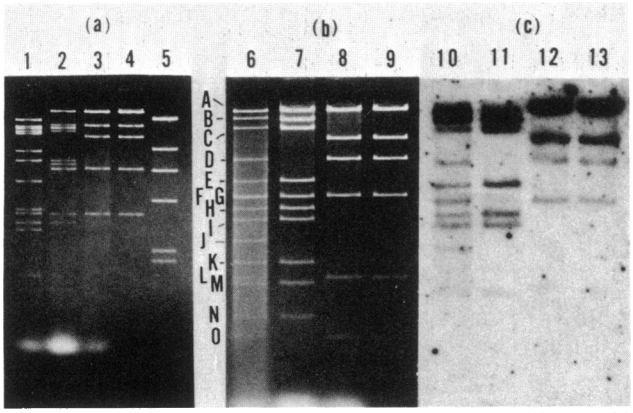


FIG. 1. *Sma*I (a) and *Xho*I (b) cleavage patterns and blot hybridization (c) patterns of pKF439, TOL(pWW0), SAL, and NAH7. Lanes: 1 and 7, TOL(pWW0); 2 and 6, pKF439; 3 and 8, SAL; 4 and 9, NAH7; 5, lambda (*Hin*dIII). Blot hybridization was done with $^{32}$P-labeled pKF439 as a probe. The *Xho*I-digested plasmids shown in (b) were blotted to nitrocellulose paper and hybridized with the labeled probe. Lanes: 10, pKF439, 11, TOL(pWW0); 12, SAL; 13, NAH7.

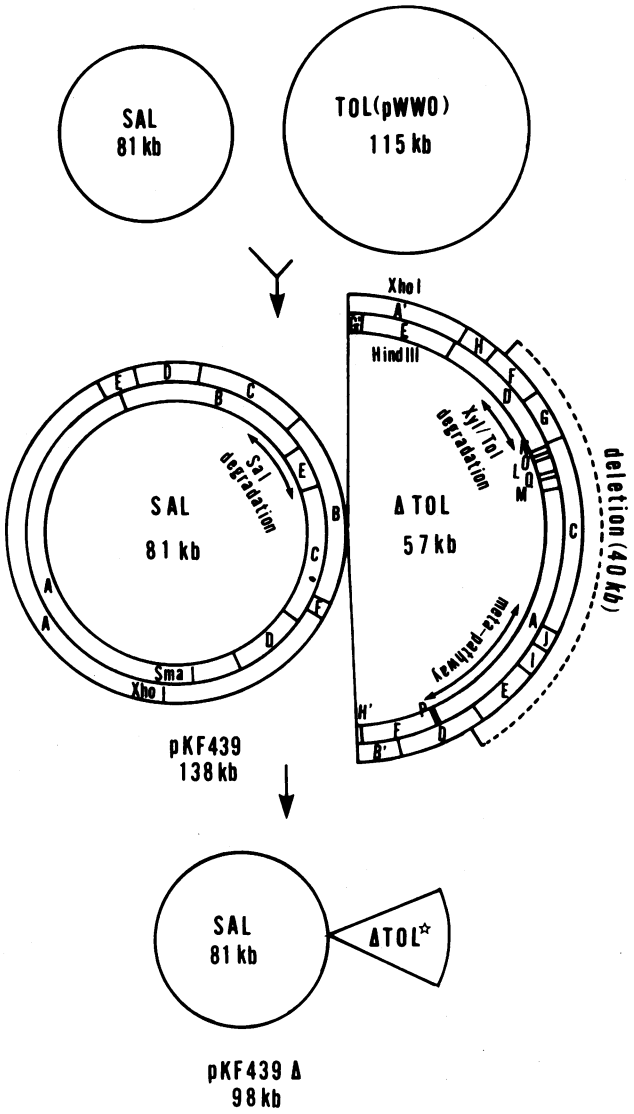


FIG. 2. In vivo rearrangement of SAL and TOL(pWW0) plasmids. The insertion site of the 57-kb TOL segment in the SAL replicon was determined from the data shown in Fig. 1. *Sma*I and *Xho*I cleavage maps of SAL DNA were determined as reported for NAH7 by Grund and Gunsalus (10). The *Xho*I and *Hin*dIII cleavage sites of the 57-kb TOL segment were labeled alphabetically as determined for the entire TOL plasmid pWW0 by Lehrbach et al. (16).

Since pKF439 has two similar catabolic sequences for catechol derived from the SAL and TOL(pWW0) genomes (Fig. 2), strain AC10(pKF439) was grown with *m*-xylene and salicylate, and oxygen uptake was measured polarographically as previously described (9). The *m*-xylene-grown cells showed high $O_2$ uptake for *m*-xylene (7.1 nmol/ml per min) and *m*-toluate (3.9 nmol/ml per min), but not for salicylate. Salicylate-grown cells showed high $O_2$ uptake only for salicylate (15.9 nmol/ml per min). Catechol 2,3-oxygenase activity was then examined for cells grown with *m*-toluate, benzoate, or salicylate. The catechol 2,3-oxygenases encoded by TOL(pWW0) and SAL were simply distinguished

by their pH optima. The pH optimum of catechol 2,3-oxygenase from TOL(pWW0) was 7.0 and from SAL was 9.0. The pH optimum of catechol 2,3-oxygenase from strain KF111(pKF439) grown with *m*-toluate or benzoate was 7.0, and that for salicylate-grown cells was 9.0. These results demonstrated that the TOL and SAL catabolic operons are independently regulated in pKF439.

In the present communication, we have demonstrated that the two IncP9 plasmids SAL and TOL(pWW0) can be stably maintained in a fused form in various strains of *P. putida* and *P. aeruginosa*. Chakrabarty (4) also indicated previously that two degradative plasmids, CAM (specifying the camphor degradation pathway) and OCT (specifying the octane degradative pathway), could be fused by UV irradiation, and the two incompatible plasmids could be stably maintained in the same cells.

TOL(pWW0) has been shown to act like a huge transposon. However, Lehrbach et al. (16) demonstrated that the fused DNA portions of TOL are not a unique DNA segment. For example, pED3304 has a continuous 69-kb region of TOL DNA inserted into a single site in RP4 within the tetracycline resistance gene. The R91-TOL hybrid plasmid pND3 contains the largest continuous fragment of TOL DNA, 104 kb. The Sa-TOL hybrid plasmid pMT101 and the RP4-TOL hybrid plasmid pTN2 contain a 56-kb segment of TOL DNA. On the other hand, pKF439, a SAL-TOL hybrid, contains a 57-kb TOL segment which is different from those of other R plasmid-TOL hybrids, but it also carries all the TOL catabolic genes. pKF439 includes and extends at both ends between a 40-kb region that is known to contain the TOL catabolic genes. This region is bounded by direct repeats and is lost when cells are grown on benzoate (17). Similarly, pKF439 lost this 40-kb region when the cells were continuously grown on benzoate (Fig. 2).

The fused TOL segment in pKF439 was inserted between *Sma*I-C and *Xho*I-B of the complete SAL replicon, so that the SAL function was not affected by the TOL DNA insertion. Williams and Worsey (22) have demonstrated that after about 20 generations of growth on benzoate, cultures of *P. putida* MT20 contained as little as 1% wild-type cells. On the other hand, pKF439 was rather stable for growth in benzoate, as in the case of SAL, and the genes responsible for replication and conjugal transfer of TOL(pWW0) are missing in pKF439, so that it is evident that the replication and conjugation ability of pKF439 is due to the functions of the SAL replicon. Moreover, it appears that the TOL and SAL catabolic operons are independently regulated in pKF439, since salicylate oxidation was stimulated only when the cells were grown on salicylate, and similarly *m*-xylene oxidation was stimulated only in *m*-xylene-grown cells. The same results were obtained from enzyme studies. Only the catechol 2,3-oxygenase gene of the TOL operon was induced when the cells were grown with *m*-toluate and benzoate. Salicylate-grown cells produced only the catechol 2,3-oxygenase of the SAL operon. Thus, it appears that there is no interaction between the TOL and SAL catabolic operons in pKF439.

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