

## Identification of Chromosomally Integrated TOL DNA in Cured Derivatives of *Pseudomonas putida* PAW1

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Some plasmid-free Tol<sup>-</sup> strains derived from *Pseudomonas putida* PAW1 (which carries the TOL plasmid pWW0) have a segment of TOL DNA located chromosomally. Of three independently isolated strains, PAW86 had an integrated TOL segment of 16 kilobases and PAW85 had two copies of this segment in different chromosomal locations, whereas the chromosomal DNA of PAW82 showed no homology with the TOL plasmid. In cultures of the parental strain, it appears that a 56-kilobase TOL DNA segment is located chromosomally in some cells.

TOL plasmid pWW0 from *Pseudomonas putida* mt-2 (strain PAW1) (Table 1) encodes the enzymes necessary for the degradation of toluene and the *m*- and *p*-xylenes via the *meta* cleavage pathway (12, 13). Strains lacking this degradative function can be selected after growth on benzoate, an intermediate in both the plasmid-encoded *meta* pathway and the chromosome-encoded *ortho* pathway (13). Such benzoate curing can occur either by loss of the plasmid from the cell or by specific excision of a 40-kilobase (kb) segment of the plasmid which results in the formation of the Tol<sup>-</sup> plasmid pWW0-8 (1). We have previously shown that the latter event occurs due to reciprocal recombination between a pair of directly repeated sequences present in the *Hind*III fragments HD and HF at the ends of the 40-kb segment. A new *Hind*III fragment, Hd, is thus formed, it being a hybrid of fragments HD and HF (8).

After the chance observation that DNA from a strain (PAW86) that had lost pWW0 after benzoate selection showed homology with the TOL plasmid in DNA-DNA hybridization studies (D. Morris and P. Broda, unpublished data), we analyzed the chromosomal DNA of strain PAW86 and two other independently isolated plasmid-free derivatives of strain PAW1 (strains PAW82 and PAW85) (Table 1) to assess the amount of pWW0 DNA present chromosomally in each.

DNA from these strains was isolated by the method of Dhaese et al (4), digested with endonuclease *Xho*I (Bethesda Research Laboratories), run out on 0.7% agarose gels, transferred

to nitrocellulose, and hybridized to pWW0 DNA that had been <sup>32</sup>P labeled by nick translation (10). DNA from the parent strain PAW1 was used as the positive control, and that from the plasmid-free strain AC34 was used as the negative control. Some homology with TOL was observed with the DNA from strains PAW85 and PAW86 but not with the DNA from strain PAW82 (Fig. 1). We next tried to establish to which segments of the TOL plasmid these regions corresponded.

Inspection of the data presented in Fig. 1 allows comparison of the homology to pWW0 observed with the DNA from strains PAW85 and PAW86 with that of the pWW0-containing strain PAW1. This shows that pWW0 segments bounded by most of the *Xho*I target sites could not be present in the DNA from the two derivative strains in the form in which they exist in pWW0 itself. We can exclude the presence of the segment that includes fragments XF, XG, XC, XJ, XI, XE, and XD. (The presence of *Hind*III fragment Hd in the chromosomal DNA of these strains excludes the possibility of *Xho*I fragment XC being present.) This continuous region includes the *Hind*III fragment HA and approximates the 40-kb segment excised by benzoate selection (Fig. 2). The presence or absence of the remainder of the pWW0 genome was tested in hybridization experiments with isolated *Hind*III-generated fragments as specific probes.

DNA samples from strains PAW82, PAW85, and PAW86 were digested with *Hind*III, run out on agarose gels, transferred to nitrocellulose filters, and hybridized to specific *Hind*III fragments of TOL. These fragments, with the exceptions of HB and HH, had been obtained as clones in the vector plasmid pBR322 (2) with

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TABLE 1. Strains used

Strain	Plasmid	Plasmid size (kb)	Cloned <i>Hind</i> III fragment <sup>a</sup>	Reference
<i>P. putida</i>				
PAW1	pWW0	115		12
PAW8	pWW0-8	75		1
PAW82, PAW85, and PAW86				1
AC34				6
<i>Escherichia coli</i>				
ED3306	pED3306	14.4	HD	8
ED3307	pED3307	11.6	HF	8
ED3309	pED3309	23.4	HC	This study
ED3310	pED3310	12.8	HE	This study
ED3311	pED3311	10.1	HG	This study
ED3312	pED3312	8.8	HI	This study
ED3313	pED3313	8.0	HJ	This study
ED3314	pED3314	6.5	HK	This study

<sup>a</sup> All *Hind*III fragments were cloned into pBR322, which is 4.3 kb (2).

*Hind*III-restricted pWW0 DNA (Table 1; R. Downing and P. Broda, unpublished data). The various probes were made by digesting 2 µg of each recombinant plasmid with *Hind*III, separating the fragments from the vector DNA on agarose gels, cutting out the gel slice containing the relevant fragment, and extracting the DNA according to the method of Thuring et al. (11). This DNA was then <sup>32</sup>P labeled by nick translation. In the cases of fragments HB and HH, the fragments were obtained after *Hind*III digestion of pWW0 DNA and subsequent separation on agarose gels.

On hybridization, DNA from strain PAW86 showed homology with probes from five *Hind*III fragments, HD (10.0 kb), HE (8.0 kb), HF (7.2 kb), HH (5.0 kb), and HK (2.7 kb). The sizes of the respective hybridizing fragments from strain PAW86 were 7.2, 6.3, 7.2, 5.0, and 1.5 kb (Fig. 3 shows the results for fragments HE and HK). No hybridization was observed with *Hind*III fragments HB, HC, HJ, HG, and HI (data not shown). The 5.0- and 7.2-kb chromosomal fragments are likely to be of purely pWW0 origin (fragments HH and Hd, respectively), but the 6.3- and 1.5-kb fragments must be composite, as would be expected if a segment of pWW0, the ends of which are located in the *Hind*III fragments HE and HK, is integrated in the chromosome of strain PAW86.

When the same experiments were performed on DNA from strain PAW85, *Hind*III fragments HD, HF, HH, HE, and HK all gave positive

results again, and negative results were obtained as with strain PAW86. The interesting observation, however, was that fragments HK and HE each showed strong homology with two *Hind*III fragments. In each case, one of the two fragments was identical in size to that hybridizing in strain PAW86 with the appropriate probe (Fig. 3). The same interpretation of the results obtained with strain PAW86 DNA is valid here except that two copies of the integrated segment appear to be present at different chromosomal

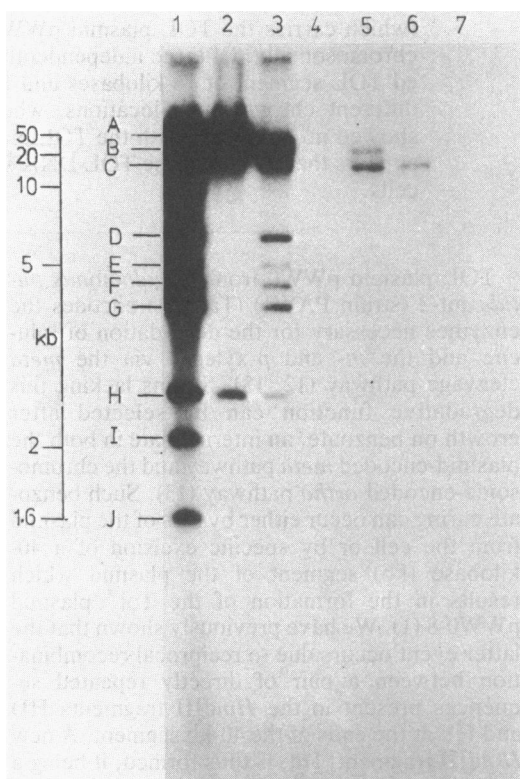


FIG. 1. Southern blots of *Xho*I-restricted chromosomal DNA with pWW0 as a labeled probe. Lanes 1 and 2 contain purified pWW0 and pWW0-8 DNA, respectively. Lanes 3 through 7 contain total cellular DNA from strains PAW1 (grown on *m*-toluate) (lane 3), PAW82 (lane 4), PAW85 (lane 5), PAW86 (lane 6), and AC34 (lane 7). In lane 6, *Xho*I fragment H of pWW0 was visible on the original autoradiogram. The different amounts of hybridization observed for certain fragments in lanes 5 and 6 reflect small differences in the quantities of DNA samples loaded on the gel. Based on the data presented in Fig. 3, two novel fragments in lane 6 should have hybridized to the pWW0 probe. The fact that only one is seen here suggests that the second may be very small, therefore running off the gel, or so large that its transfer onto the filter would be very inefficient. In lane 3, bands XG and XD are more intense due to the repeated DNA sequence present within *Hind*III fragments HD and HF.

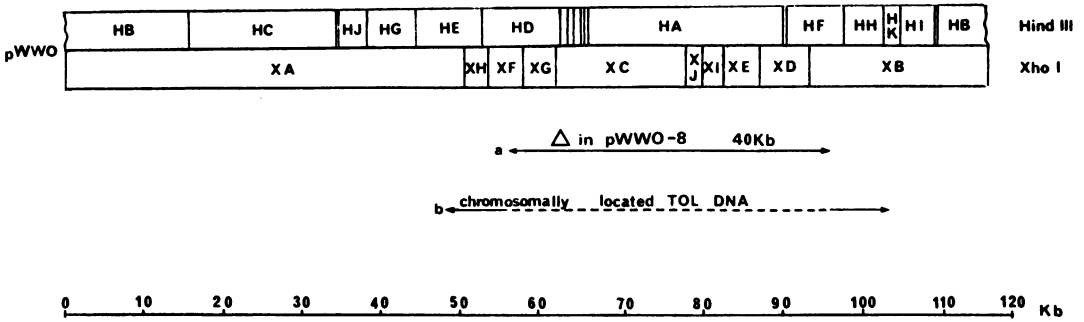


FIG. 2. *HindIII* and *XhoI* cleavage maps of pWW0 showing the specific deletion occurring in pWW0-8 formation (a) and the endpoints of the integrated segment in strains PAW85 and PAW86 (b). The *XhoI* cleavage map differs from that previously published (5) in that the positions of fragments XE and XI are reversed. The dotted portion of line (b) indicates the DNA which is absent in the cured derivative strains PAW85 and PAW86. This corresponds in size and location to the 40-kb excised segment represented by line (a). The solid portion of line (b) shows the DNA which is present in the cured derivatives.

locations; one would be at the same site as in strain PAW86.

As stated above, *HindIII* digests of total cellular DNA from strain PAW1 were routinely included as positive controls in the different hy-

bridizations with the specific *HindIII* fragments as probes. In general, the expected results were obtained; namely, homology was observed only with the single fragment used as the probe. However, in the cases of fragments HE and HK, homology to additional fragments (6.3 and 1.6 kb, respectively) was observed. These fragments are not carried by the pWW0 plasmid itself, and we conclude that in a significant proportion of cells of our culture of the parental strain, PAW1, there is also pWW0 DNA at a particular chromosomal location.

This segment could be a continuous region of the pWW0 genome containing all of the DNA sequences between *HindIII* fragments HK and HE (56 kb). However, it was not possible to demonstrate this by the experiments described here. Such a model in turn suggests that the integrated segment in strains PAW85 and PAW86 is the 16 kb of DNA that remains after the excision of the internal 40-kb portion of the 56-kb segment. The results obtained with strain PAW85 suggest that a second copy of this region can be maintained at a second location. The lack of any pWW0 DNA in the chromosome of strain PAW82 could be explained by the straightforward loss of the entire plasmid in this particular curing event.

A similar segment of 56 kb of pWW0 DNA has

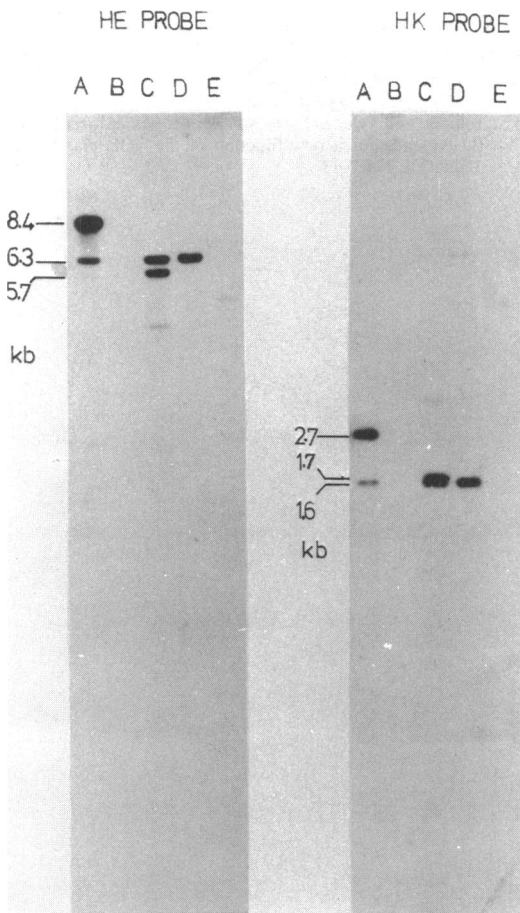


FIG. 3. Southern blots of *HindIII*-restricted chromosomal DNA with fragments HE and HK as probes. Lanes: A, strain PAW1; B, strain PAW82; C, strain PAW85; D, strain PAW86; E, strain AC34. The strongly hybridizing band in lane C of the HK probe is a doublet. The faint bands visible in lanes C and E of the HE probe and lanes C and D of the HK probe may reflect some homology between the probes and more distantly related chromosomal sequences; however, we cannot discount the possibility that this observation is due to nonspecific hybridization.

been implicated in recombination events which superficially resemble transposition involving TOL and plasmids RP4 (9), Sa (P. R. Lehrbach, J. M. Ward, P. Meulien, and P. Broda, *J. Bacteriol.*, in press), and R2 (7). Evidence that a toluene-degrading pathway could have a chromosomal location comes from the existence of *P. putida* PAM1, a strain which has the same origin as strain PAW1 and is phenotypically Tol<sup>+</sup>. However, it harbors a plasmid identical to pWWO-8, which can be expelled from the cell without the loss of the Tol<sup>+</sup> function (7).

(A preliminary report of some of these results was presented at a symposium in Santo Domingo in January 1981 [3].)

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