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Spontaneous Deletion of a 20-Kilobase DNA Segment Carrying Genes Specifying Isopropylbenzene Metabolism in *Pseudomonas putida* RE204

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The genes encoding isopropylbenzene metabolism in *Pseudomonas putida* RE204 are readily lost in two ways: (i) by loss (curing) of plasmid pRE4 which specifies the catabolic pathway and (ii) by deletion from pRE4 of an approximately 20-kilobase segment of DNA carrying the catabolic genes. The presence of DNA sequences at the ends of the catabolic gene region sharing homology with one another suggests that the deletions result from recombination events between these homologous sequences.

Pseudomonas putida RE204 grows with isopropylbenzene (Ipb) as the sole carbon and energy source, and this metabolic ability is encoded on a 105-kilobase (kb) plasmid named pRE4 (6). A transposon insertion and restriction enzyme cleavage map of the region of DNA encoding isopropylbenzene catabolism has been obtained (6), and a simplified version of this region is shown in Fig. 1. In addition to Tn*5*-generated mutants, two types of mutants which lack the plasmid-carried catabolic genes were isolated.

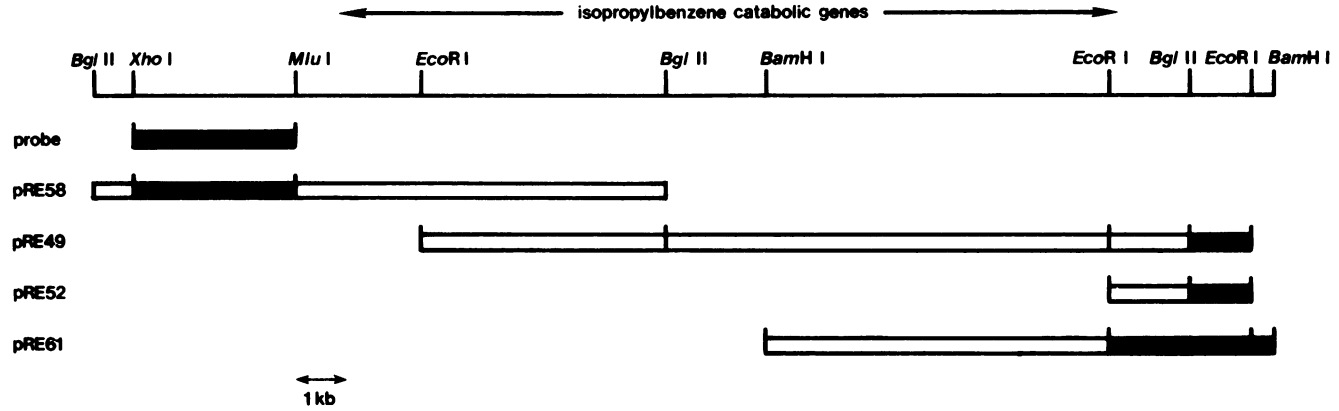

FIG. 1. Isopropylbenzene metabolic gene region of pRE4. Shown are relevant restriction enzyme cleavage sites (not all *Eco*RI, *Mlu*I, and *Bam*HI sites are shown) and the location of the isopropylbenzene metabolic genes. Below are indicated the segments of the plasmids used in the Southern hydribization experiment (Fig. 2) which were derived from pRE4. The filled portions of these segments hybridized with the *Xho*I-*Mlu*I fragment probe at the left.

Isolation of mutants lacking isopropylbenzene catabolic genes. *P. putida* RE204 was grown at 34°C for 40 generations in LB medium (4) and streaked on LB agar for single colonies, and the colonies that developed were tested for growth on minimal medium supplemented with fumarate or isopropylbenzene (6). In four separate experiments a total of 664 colonies were tested; all grew on fumarate, but only 31 of these grew on isopropylbenzene. Using the procedure of Birnboim and Doly (2), which normally reveals the pRE4 plasmid in strain RE204, two Ipb$^-$ strains from each experiment were checked for plasmid DNA; all were found to lack pRE4.

During several Tn*5* mutagenesis experiments with *P. putida* RE204 in which about 14,000 Tn*5*-containing derivatives were tested, Ipb$^-$ clones (at least 16 strains from five experiments) were isolated that were subsequently found to have suffered deletions in the pRE4 plasmid. Digestion of plasmid DNA isolated from these strains by the method of Hansen and Olsen (7) with several restriction endonucleases (*Eco*RI, *Xho*I, *Mlu*I) indicated that these deletions were identical (data not shown) and extended from a point in the *Mlu*I-*Xho*I fragment at the left of the map shown in Fig. 1 to a point about 20 kb to the right which would be in or just past the 2.7-kb *Eco*RI fragment at the right of the map. This conclusion is based on the fact that the *Xho*I site was retained in the deletion derivative plasmids, whereas the *Mlu*I site was not; the size of the deleted fragment was calculated from the sum of the sizes of fragments produced by digestion with *Eco*RI and with *Mlu*I.

Apparently identical deletion derivative plasmids were isolated by growing *P. putida* RE204 at 30°C for 40 to 50

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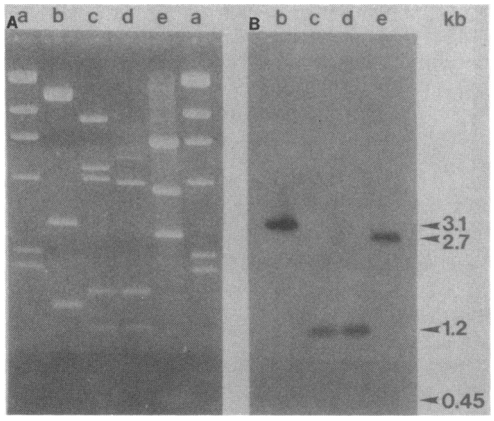

FIG. 2. Demonstration of regions of homology flanking the isopropylbenzene metabolism-encoding region of pRE4 by Southern blot analysis. (A) Lanes: a, *Hin*dIII-digested bacteriophage λ DNA; b, *Mlu*I-*Xho*I-digested pRE58; c, *Eco*RI-*Bgl*II-digested pRE49; d, *Eco*RI-*Bgl*II-digested pRE52; and e, *Bam*HI-*Eco*RI-digested pRE61. (A) Ethidium bromide-stained gel; (B) autoradiogram of the Southern blot probed with the ^{32}P-labeled 3.1-kb *Xho*I-*Mlu*I fragment.

generations and then checking for loss of the Ipb phenotype. In one experiment, 4 Ipb$^-$ clones were isolated of 270 tested, and all these had suffered a 20-kb deletion in pRE4.

Because a considerable number of independently isolated deletion derivatives of pRE4 seemed to be identical, they might have resulted through recombination between homologous DNA sequences located at the ends of the DNA segments deleted. This possibility was tested by Southern hybridization. Recombinant plasmids carrying portions of the isopropylbenzene catabolic region (Fig. 1) (6) were digested with restriction endonucleases and electrophoresed through a 0.7% agarose gel as previously described (6). After the gel was stained with ethidium bromide and photographed (Fig. 2A), the DNA was transferred to a nitrocellulose filter (15). This filter was hybridized (17) with the 3.1-kb *Xho*I-*Mlu*I fragment which had been purified by cleavage of pRE58 DNA with *Xho*I and *Mlu*I, agarose gel electrophoresis of the fragments, and elution of the 3.1-kb fragment from the gel (5) and was subsequently ^{32}P labeled by nick translation (9). The filter was washed in 2× SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C and placed on X-ray film with intensifier screens at −70°C. The autoradiogram (Fig. 2B) shows that the *Xho*I-*Mlu*I fragment probe hybridized to itself and to a 1.2-kb *Bgl*II-*Eco*RI fragment at the right-hand end of the map in Fig. 1. The adjacent 0.45-kb *Eco*RI-*Bam*HI fragment also hybridized to the probe, but this is not readily apparent in Fig. 2B. The probe did not hybridize to the 17 kb of DNA between the *Mlu*I site and the *Bgl*II site.

The regions of homology flanking the isopropylbenzene catabolic genes are reminiscent of the repeated sequences found at the ends of transposable elements, some of which carry genes specifying metabolic functions (10). To test for the ability of the catabolic gene region to transpose, the broad-host-range conjugative plasmids RP4 (16) and Sa (18) were separately introduced into *P. putida* RE204 and then transferred out into a derivative of RE204 that was cured of pRE4 and resistant to rifampin and nalidixic acid. Transconjugants were plated on minimal medium containing rifampin, nalidixic acid, and kanamycin and either fumarate or isopropylbenzene. Transfer of RP4 and Sa occurred but at frequencies too low to have allowed detection of transposition if it had occurred.

The results reported here are similar to those obtained with the TOL plasmid from which a 39-kb segment encoding toluate metabolism is readily deleted (1, 3) as the result of recombination between 1.4-kb directly repeated sequences located at the ends of the segment (13). Although this region of TOL has been shown to transpose to other replicons (3, 8, 14, 19), this transposition apparently doesn't involve these repeated sequences or a unique segment of DNA because the transposed TOL-derived sequences vary in size and extend beyond the 39-kb segment at both ends (11, 12).

In conclusion, the isopropylbenzene catabolic genes are lost from *P. putida* RE204 in two ways: either by loss of the plasmid pRE4 or by deletion of an isopropylbenzene catabolic gene-carrying DNA fragment from pRE4. This deletion probably results from recombination between homologous DNA sequences which bracket the catabolic genes. Although these homologous sequences may constitute the ends of a large transposon, no evidence for transposability of the DNA segment was obtained.

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