Identification of *Pseudomonas alcaligenes* Chromosomal DNA in the Plasmid DNA of the Chlorobenzene-Degrading Recombinant *Pseudomonas putida* Strain CB1-9

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The recombinant *Pseudomonas putida* strain CB1-9, which acquired the ability to grow on chlorobenzenes, contains a 33-kilobase (kb) plasmid (pKFL3) which lacked homology to an indigenous 15-kb plasmid (pKFL1) in *Pseudomonas alcaligenes* C-0 parent but was homologous to a 55-kb plasmid (pKFL2) from the *P. putida* R5-3 parent. Chromosomal DNA of *P. alcaligenes* C-0 hybridized to probes prepared from pKFL3 but not to probes prepared from pKFL2. A single clone from a genomic library of *P. alcaligenes* C-0 hybridized to EcoRI-digested pKFL3. Southern blot hybridization with the insert DNA from that clone identified homology with specific restriction enzyme fragments in pKFL3. The ability of the recombinant to utilize 3-chlorobenzoate, chlorobenzene, and 1,4-dichlorobenzene as well as its loss of utilization of xylenes and methylbenzoates appears to be associated with the transfer and integration of chromosomal DNA from *P. alcaligenes* into a Tol-like plasmid of *P. putida* R5-3.

Recently, Kröckel and Focht (5) reported the construction of a recombinant *Pseudomonas putida* strain, CB1-9, by a novel continuous amalgamated culture method. Two parental strains, *P. putida* R5-3 and *Pseudomonas alcaligenes* C-0 (3), were continuously mated under chlorobenzene selection pressure and gave rise to the recombinant *P. putida* CB1-9. The *P. putida* R5-3 parent was able to grow on a range of aromatic hydrocarbons, while the *P. alcaligenes* C-0 parent was capable of metabolizing 3-chlorobenzoate as the sole carbon source. Unlike either parent, strain CB1-9 had the ability to metabolize toluene, 3-chlorobenzoate, chlorobenzene, and 1,4-dichlorobenzene as sole carbon sources, indicating that it had acquired phenotypes from both parental strains in addition to the novel phenotype. The work presented here determined the genetic contribution of both parents in the construction of the new recombinant strain CB1-9.

**Plasmid DNA and restriction enzyme analysis.** Each of the parent strains and the recombinant strain were found to have a single plasmid (5); *P. alcaligenes* C-0 contained a small 15-kilobase (kb) plasmid designated pKFL1, while *P. putida* R5-3 had a large 55- to 60-kb plasmid designated pKFL2. The recombinant *P. putida* strain CB1-9 contained a 33-kb plasmid designated pKFL3 which was not observed in either of the parental strains.

Restriction enzyme digest analysis (6) with EcoRI, BglII, and EcoRI-BglII revealed that pKFL2 shared common fragments with pKFL3. No common restriction enzyme fragments were observed in pKFL1 and pKFL3 or in pKFL1 and pKFL2. Novel EcoRI fragments in pKFL3 (7.5 and 1.8 kb) were not present in either pKFL1 or pKFL2.

**Nucleic acid hybridization.** An EcoRI digest of pKFL3 was used as a probe in Southern blot hybridizations (9) with EcoRI digests of pKFL1, pKFL2, and pKFL3. pKFL3 hybridized to itself and to several common restriction fragments in pKFL2 (Fig. 1). No homology was observed between pKFL3 and pKFL1, either at high stringency or on repeating under low-stringency hybridization conditions.

Because of the absence of homology between pKFL3 and the plasmid DNA of the *P. alcaligenes* parental strain (pKFL1) and the presence of the unique EcoRI fragments (Fig. 1, lane 4b), further hybridizations were carried out to investigate the possibility of chromosomal-DNA involvement. EcoRI digests of pKFL1, pKFL2, and pKFL3 were used as probes in three separate hybridizations with undigested plasmid and chromosomal DNAs of strains C-0, R5-3, and CB1-9 (Fig. 2). The pKFL3 plasmid DNA of *P. putida* CB1-9 hybridized with both plasmid and chromosomal DNAs of C-0 and of the *P. putida* parent, R5-3, and with only chromosomal DNA of the parent *P. alcaligenes* C-0. When pKFL2 was used as a probe, homology was observed with plasmid and chromosomal DNAs of R5-3 and CB1-9. The hybridization of *P. putida* and *P. alcaligenes* plasmid DNAs with their own cesium-purified chromosomal DNAs is most likely due to the inability to separate the chromosomal DNA from nicked plasmid DNA during purification. In the third hybridization, with pKFL1 as probe DNA, homology only to itself and to C-0 chromosomal DNA was observed.

**P. alcaligenes genomic library screening.** A genomic library of *P. alcaligenes* C-0 was constructed to verify that chromosomal DNA had been incorporated into pKFL3. When an EcoRI digest of pKFL3 was used as a probe in colony hybridization (4) to the library clones, five possible positive clones were identified. Subsequent Southern blot hybridization using the same probe with purified plasmid DNA from these clones identified only one (clone 227) as positive. The hybridization using the EcoRI-HindIII fragments of the *P. alcaligenes* chromosomal DNA from clone 227 as probe indicated that the probe DNA hybridized strongly with specific restriction enzyme fragments of pKFL3 (Fig. 3).

DNA-DNA hybridization studies with pKFL3 as probe DNA identified homologous sequences in the chromosomal DNA of *P. alcaligenes* and in the pKFL2 plasmid DNA of *P. putida* R5-3 (Fig. 2). Hybridizations with either pKFL1 or

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pKFL2 as probes showed no homology between the two parental strains, in either plasmid or chromosomal DNA. In addition, pKFL1 was not homologous to either plasmid or chromosomal DNA of *P. putida* CB1-9, while pKFL2 hybridized strongly to the plasmid DNA of the new recombinant. This supports previous observations based on physiology and phenotypes (5) that CB1-9 is more closely related to *P. putida* R5-3 than to *P. alcaligenes* C-0. These results indicate that the acquisition of the ability to degrade 3-chlorobenzoate, chlorobenzene, and 1,4-dichlorobenzene by *P. putida* CB1-9 was achieved by the integration of chromosomal DNA from *P. alcaligenes* C-0 into pKFL2 (the plasmid DNA of *P. putida* R5-3), resulting in the recombinant plasmid pKFL3 in *P. putida* CB1-9.

Since chlorobenzene and 1,4-dichlobenzene are both co-metabolized to the corresponding chlorocatechols by the parental recipient, *P. putida* R5-3, without release of chloride, the genes from the parental donor, *P. alcaligenes* C-0, clearly must code for enzymes downstream from chlorocatechols in the metabolic pathway. Because the recombinant *P. putida* CB1-9 has acquired the ability to utilize 3-chlorobenzoate as the sole carbon source, which is metabolized through an *ortho* fission of 3-chlorocatechol, it is conceivable that all of the genes coding for the part of the pathway from 3-chlorocatechol to acetate and succinate may be present in the chromosomal fragment transferred from C-0 to R5-3.

All attempts to cure the recombinant *P. putida* CB1-9 of the 33-kb plasmid containing the *P. alcaligenes* C-0 chromosomal DNA insert by prolonged growth on benzoate or in rich medium at 33°C with or without ethidium bromide were unsuccessful. Therefore, the role of the fragment of *P. alcaligenes* chromosomal DNA in conferring the ability of *P. putida* CB1-9 to utilize 3-chlorobenzoate as the sole carbon source was confirmed by transposon mutagenesis using the Tn5 donor *Escherichia coli* S17 carrying pUW964, selecting for kanamycin resistance, and screening for the inability to utilize 3-chlorobenzoate. Two such mutants were selected, and the 33-kb plasmid was isolated and hybridized to the internal HindIII fragment of Tn5. A single copy of Tn5 was detected in the plasmid DNA and localized to a 7.5-kb EcoRI fragment known to consist entirely of *P. alcaligenes* chromosomal DNA.

Triparental matings (2) were performed between the transposon mutants, *E. coli* HB101 (carrying the mobilizing plasmid pRK2013) and the cosmid library clone 227. Each of the transconjugants recovered the ability to utilize 3-chlorobenzoate as the sole carbon source.

Restriction enzyme digests of pKFL3 compared with those of pKFL2 revealed multiple common restriction frag-
ments, the absence of other fragments, and the presence of novel fragments not observed in pKFL2. Southern blot hybridizations confirmed that the common fragments in pKFL2 and pKFL3 were indeed homologous to each other (Fig. 2) and that the unique fragments of pKFL3 were due to the incorporation of P. alcaligenes chromosomal DNA into pKFL2 (Fig. 3). It is thus apparent that pKFL3 is a derivative of pKFL2 in which deletions, rearrangements, and recombination events have taken place, resulting in a new 33-kb plasmid.

The inability of P. putida CB1-9 to grow on methylbenzoates or xylenes (5) may be related to partial loss of the meta-fission pathway (7) and the corresponding loss of approximately 30 kb of DNA in the derivation of pKFL3. In the construction of a 3-chlorobenzoate- and 3,5-dichlorobenzoate-degrading Pseudomonas sp. by introducing Tol genes into a 3-chlorobenzoate utilizer (1, 8), a similar phenomenon was observed in that prevention of the meta cleavage of chlorocatechols allowed degradation of new substrates (7). There is strong evidence that pKFL2 and derived forms of pKFL2 are indeed Tol-like plasmids (B. F. Carney and J. V. Leary, manuscript submitted for publication) and that the introduction of 3-chlorobenzoate genes into P. putida R5-3 caused the inactivation or deletion of genes for the meta cleavage enzymes.

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