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Genes specifying degradation of 3-chlorobenzoic acid in plasmids pAC27 and pJP4

(genome organization/gene cloning/gene amplification/genetic rearrangements/repetitive sequences)

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ABSTRACT **All of the structural genes for 3-chlorobenzoate degradation are clustered in a 4.2-kilobase (kb) region of plasmid pAC25 (or pAC27) in *Pseudomonas putida*. An approximate 10-kb DNA segment containing three structural genes for chlorocatechol metabolism present on plasmid pJP4 in *Alcaligenes eutrophus* shows homology with the above 4.2-kb region of pAC27. In spite of the detectable sequence homology in the structural genes present on both plasmids, the regulation of their expression seems quite different; unlike pAC27, structural rearrangements are prerequisite for efficient expression of the 3-chlorobenzoate genes on plasmid pJP4. Structural features such as stem–loop structures present on plasmid pJP4 are most likely the starting materials for such rearrangements.**

Complete degradation of 3-chlorobenzoate by a soil bacterium such as *Pseudomonas putida* is specified by plasmid pAC27 (a deletion derivative of pAC25) or pWR1. Plasmids pAC27 and pWR1 (pB13) have extensive homology and are probably identical (1). Plasmid pJP4 present in *Alcaligenes eutrophus* (2) encodes resistance to mercury and degradation of 3-chlorobenzoate and 2,4-dichlorophenoxyacetic acid (2,4-D). The complete pathway for 3-chlorobenzoate degradation, the enzymes involved in the sequential steps, the nature of enzymes encoded by plasmids pWR1 and pJP4, as well as the role of chromosomal genes have been reviewed (3).

Insertional mutation analysis of pJP4 using Tn*5* and subsequent detection of intermediates accumulated during 3-chlorobenzoate catabolism by these mutants have shown that the three genes specifying chlorocatechol degradation are clustered in a 3-kilobase (kb) region within the *Eco*RI B fragment (3). These catabolic genes have not been localized on pAC27 (or pWR1). Furthermore, very little is known about the regulation of 3-chlorobenzoate gene expression in both pJP4 and pAC27. In this paper we present evidence that these degradative genes are clustered in plasmids pJP4 and pAC27, and the efficient expression of 3-chlorobenzoate degradative genes in plasmid pJP4 occurs only after substantial genetic rearrangement in the plasmid, presumably via repetitive sequences that are present on plasmid pJP4 in both direct and inverted orientations.

MATERIALS AND METHODS

The relevant properties of strains used are described in Table 1. Media, conditions for the cultivation of strains, isolation and analysis of plasmids, mobilization of conjugative and non-conjugative plasmids, and Southern hybridization analysis have already been described (1, 2, 4). Electron microscopy of DNA was carried out according to Ohtsubo and Ohtsubo (7). Cloning of DNA fragments to cosmid vector pLAFR1 or plasmid vector pRK290 was carried out in *Escherichia coli* AC80 by transfection or transformation followed by mobilization to *P. putida* for the phenotypic expression of the degradative genes (4).

Table 1. Bacterial strains and plasmids

Strain	Species	Plasmid	Phenotype	Ref.
JMP134	*A. eutrophus*	pJP4	2,4-D$^+$, 3Cba$^+$, Hg^r	2
AC80	*E. coli*	None	Thr$^-$ Leu$^-$ Met$^-$ HsdR$^-$	4
Y43	*E. coli*	pY43	Thr$^-$ Leu$^-$ Met$^-$ HsdR$^-$ HsdM$^-$	This study
AC867	*Pseudomonas aeruginosa*	pAC27	3Cba$^+$ 4Cba$^+$	1
DC10	*P. putida*	pDC10	3Cba$^+$ Tc^r	This study
DC15	*P. putida*	pDC15	3Cba$^+$ Tc^r	This study
DC20	*P. putida*	pDC20	3Cba$^+$ Tc^r	This study
DC25	*P. putida*	pDC25	3Cba$^+$ Tc^r	This study
PpG1900	*P. putida*	None	Km^r Trp$^-$	5
YG2	*P. putida*	pYG2	3Cba$^+$ Km^r Trp$^-$ Hg^r	This study
YG410	*P. putida*	pYG410	3Cba$^+$ Km^r Tc^r Trp$^-$	This study
YG419	*P. putida*	pYG419	3Cba$^+$ Tc^r Km^r Trp$^-$	This study
YG1943	*P. putida*	pYG1943	3Cba$^+$ Tc^r Km^r Trp$^-$	This study
383	*Pseudomonas cepacia*	None		6

3Cba = 3-chlorobenzoate; Hg^r = mercury resistance; Tc^r = tetracycline resistance; Km^r = kanamycin resistance; Trp$^-$, requires tryptophan.

RESULTS

Cloning of the 3-Chlorobenzoate Genes from Plasmids pAC27 and pJP4. Cloning of genes specifying 3-chlorobenzoate degradation in *P. putida* was carried out by using the broad host range vectors pLAFRI (for *Eco*RI fragments) and pRK290 (for *Bgl* II fragments). The vector DNA and pAC27 DNA were digested with either *Eco*RI or *Bgl* II, ligated, packaged in phage λ, and introduced into *E. coli* cells by transfection (for cosmid pLAFRI) or transformation (for pRK290). Recombinant plasmids containing the 3-chlorobenzoate genes were transferred to *P. putida* by conjugation en masse, selecting on minimal 3-chlorobenzoate agar. Restriction endonuclease analysis of various 3-chlorobenzoate-positive clones obtained from plasmid pAC27 and a physical map of pAC27 are shown in Fig. 1 *b* and *a*, respectively. Plasmid pDC10 contains the vector pLAFRI and the *Eco*RI

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Abbreviations: kb, kilobase(s); 2,4-D, 2,4-dichlorophenoxyacetic acid.

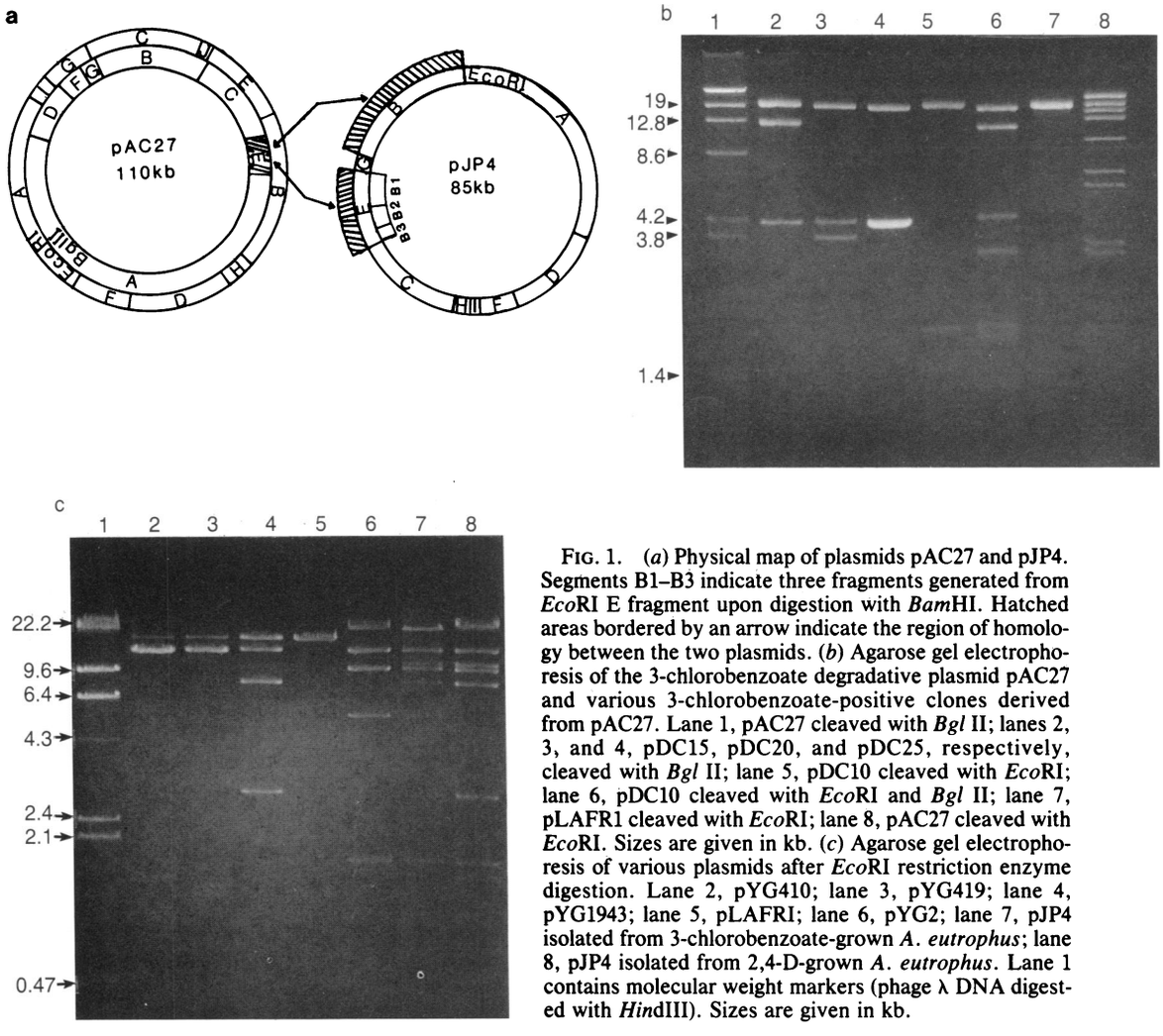

FIG. 1. (*a*) Physical map of plasmids pAC27 and pJP4. Segments B1–B3 indicate three fragments generated from *Eco*RI E fragment upon digestion with *Bam*HI. Hatched areas bordered by an arrow indicate the region of homology between the two plasmids. (*b*) Agarose gel electrophoresis of the 3-chlorobenzoate degradative plasmid pAC27 and various 3-chlorobenzoate-positive clones derived from pAC27. Lane 1, pAC27 cleaved with *Bgl* II; lanes 2, 3, and 4, pDC15, pDC20, and pDC25, respectively, cleaved with *Bgl* II; lane 5, pDC10 cleaved with *Eco*RI; lane 6, pDC10 cleaved with *Eco*RI and *Bgl* II; lane 7, pLAFR1 cleaved with *Eco*RI; lane 8, pAC27 cleaved with *Eco*RI. Sizes are given in kb. (*c*) Agarose gel electrophoresis of various plasmids after *Eco*RI restriction enzyme digestion. Lane 2, pYG410; lane 3, pYG419; lane 4, pYG1943; lane 5, pLAFRI; lane 6, pYG2; lane 7, pJP4 isolated from 3-chlorobenzoate-grown *A. eutrophus*; lane 8, pJP4 isolated from 2,4-D-grown *A. eutrophus*. Lane 1 contains molecular weight markers (phage λ DNA digested with *Hin*dIII). Sizes are given in kb.

B and J fragments of pAC27 (Fig. 1*b*, lane 5). Because of similar sizes (21 kb), the fragment *Eco*RI B and linearized pLAFRI are not resolved on the gel. The *Eco*RI B fragment contains part of *Bgl* II A and *Bgl* II C and complete E fragments, as shown by double digestion of plasmid pDC10 with *Eco*RI and *Bgl* II (Fig. 1*b*, lane 6). Plasmid pDC15 isolated from clone DC15 shows the presence of a 17-kb DNA insert that contains two contiguous *Bgl* II fragments, *Bgl* II E and *Bgl* II C of pAC27 (Fig. 1*b*, lane 2). Plasmid pDC20 isolated from clone DC20 contains two *Bgl* II fragments, *Bgl* II E and *Bgl* II F of pAC27 (Fig. 1*b*, lane 3). Plasmid pDC25 isolated from DC25 shows the presence of the single 4.2-kb *Bgl* II E fragment besides the vector DNA (Fig. 1*b*, lane 4), suggesting that three structural genes specifying chlorocatechol degradation and the promoter sequences for the expression of these genes are present on this fragment. The presence of operator–promoter sequences in this fragment is indicated by the fact that maximal 3-chlorobenzoate oxidation and chloride release were obtained only when DC25 cells were grown in presence of 3-chlorobenzoate. Growth of clone DC25 in the presence of succinate did not elicit 3-chlorobenzoate oxidation by resting cells and benzoate-grown cells released only 25% of the chloride normally released by 3-chlorobenzoate-grown resting cells during 4 hr of incubation. The overlapping region between pDC10 or pDC15 (*Bgl* II E plus part of *Bgl* II C) is likely to encode additional function for rapid 3-chlorobenzoate degradation, since the two clones DC20 and DC25 appeared on minimal 3-chlorobenzoate selective plates after a long lag and grew somewhat slower than DC15.

Cloning of 3-chlorobenzoate genes from plasmid pJP4 was carried out the same way as with pAC27. In addition to en masse mobilization, recombinant plasmids of known combination were individually transferred to *P. putida* and selected for 3-chlorobenzoate degradation and tetracycline resistance ($3Cba^+$ Tc^r) phenotype. Among various combinations tested, a clone containing the pJP4 fragments *Eco*RI B, *Eco*RI E, and *Eco*RI F ligated with pLAFRI gave rise to $3Cba^+$ Tc^r colonies upon mobilization, and this *E. coli* clone was designated as Y43. Restriction endonuclease analysis of plasmid from 3-chlorobenzoate-positive clones (YG410 and YG419) obtained from en masse mobilization and a physical map of plasmid pJP4 are shown in Fig. 1 *c* and *a*, respectively. Clone YG410 contained *Eco*RI B and *Eco*RI H fragments, whereas clone YG419 contained *Eco*RI B fragment only (Fig. 1*c*, lanes 2 and 3). Clone YG1943 obtained from the mobilization of plasmid pY43 from *E. coli* Y43 into *P. putida* PpG1900 showed the presence of fragments *Eco*RI B, *Eco*RI E, and *Eco*RI F (Fig. 1*c*, lane 4). Clones YG410 and YG419 grew slower on 3-chlorobenzoate and produced brown coloration during growth with 3-chlorobenzoate, whereas clone YG1943 grew rapidly without producing any colored product. The location of these fragments on the physical map of pJP4 is shown in Fig. 1*a*.

Amplification of Cloned 3-Chlorobenzoate Genes. Contour length measurement of plasmid DNAs isolated from clones DC25 and YG419 grown on 3-chlorobenzoate shows average molecular sizes of 60 kb and 120 kb, respectively. Restriction endonuclease digestion of pDC25 and pYG419 (from 3-chlorobenzoate-grown cells) with *Bgl* II (Fig. 1*b*, lane 4) and

*Eco*RI (Fig. 1*c*, lane 3) generates only two fragments, vector pRK290 (20 kb) and a 4.2-kb insert, in the case of pDC25 and pLAFRI (21 kb) and a 15-kb insert in the case of pYG419. In both cases in agarose gel the insert bands show much higher intensity compared to that of the vector band. These plasmids show the absence of "snap-back" structures under the electron microscope upon denaturation and fast renaturation (not shown). This appears to suggest the presence of tandem amplifications of *Bgl* II E fragment (9 or 10 copies per plasmid) in the case of pDC25 and *Eco*RI B fragment (about 7 copies per plasmid) in pYG419. Contour length measurement of plasmid DNAs isolated from glucose/tetracycline-grown cells of YG419 or DC25 shows an average molecular size of 35–40 kb or 25 kb—i.e., only pLAFRI with a single copy of the *Eco*RI B or pRK290 with a single copy of the *Bgl* II E insert. This suggests that the presence of *Bgl* II E fragment of pAC27 and *Eco*RI B fragment of pJP4, which contain all of the structural genes for chlorocatechol degradation in *P. putida*, was not sufficient to impart 3-chlorobenzoate phenotype readily without amplification or genetic rearrangements. This presumably indicates the presence of positive regulatory element(s) outside the cloned fragment. Absence of this element may necessitate a gene dosage effect through amplification because of the strong selection pressure to allow growth on 3-chlorobenzoate. As expected, the amplified plasmid DNAs and the resulting phenotype are more stable in cells having mutation in the host *recA* system. Detailed results of the effect of the host recombination system in the amplification of this gene cluster will be published elsewhere.

Southern Hybridization Between Various Restriction Fragments of Plasmids pJP4 and pAC27. Since both plasmids pJP4 and pAC27 encode the genes for chlorocatechol degradation, their DNA sequence homology was determined by Southern hybridization. *Eco*RI fragments B, E, and F of pJP4 were individually purified, nick-translated, and used as probes to hybridize with *Eco*RI and *Bgl* II-digested pAC27 DNA. pJP4 fragment *Eco*RI F did not show any homology, whereas *Eco*RI B and *Eco*RI E fragments showed significant homology with the 4.2-kb *Bgl* II E fragment of pAC27 (6). Control experiments with *Eco*RI fragments A, C, D, and G of pJP4 did not show any hybridization with any of the pAC27 fragments. In a parallel experiment, the same 4.2-kb *Bgl* II E fragment of pAC27 was purified and used as a probe to check for homology with *Eco*RI or *Eco*RI plus *Bam*HI-digested pJP4 (6). The homology was restricted to a 10-kb region of *Eco*RI B and proximal 3-kb region of *Eco*RI E of pJP4. The results of this homology study are summarized in Fig. 1*a*. These results in combination with the cloning data suggest that all three structural genes needed for chlorocatechol degradation are present on the *Eco*RI B fragment. The homologous segments between the two plasmids are continuous in pAC27 as part of the 4.2-kb *Bgl* II E fragment, whereas they are interrupted by at least a 2-kb fragment (*Eco*RI G) in pJP4 (see Fig. 1*a*).

DNA Sequence Rearrangements of pJP4 in 3-Chlorobenzoate-Positive *P. putida*. *A. eutrophus* JMP134 cells (harboring pJP4) routinely maintained on 2,4-D-containing medium do not readily express 3-chlorobenzoate phenotype. The expression of the 3-chlorobenzoate pathway encoded by plasmid pJP4 was investigated after conjugal transfer of the plasmid to *P. putida* and *P. cepacia* by selecting the exconjugants on *Pseudomonas* isolation agar containing merbromin. Although when selected for Hg^r, the $pJP4^+$ transconjugants were isolated in *P. putida* at a frequency of 10^{-2} to 10^{-3}, selection on 3-chlorobenzoate allowed recovery of exconjugants at a frequency of 10^{-7} or below. Furthermore, it takes 4–5 weeks for the transconjugants to express the 3-chlorobenzoate degradative phenotype efficiently.

Five 3-chlorobenzoate-positive *P. putida* clones designated YG2 to YG6 from two independent conjugation experiments with *A. eutrophus* harboring pJP4 as donor were selected. The restriction endonuclease profiles of the plasmids isolated from these clones were identical. All of the plasmids demonstrated considerable DNA rearrangements in comparison to the parental plasmid pJP4 involving gene duplication followed by deletion and fusion. The *Eco*RI fragmentation pattern of pYG2 (Fig. 1*c*, lane 6) and the molecular sizes of the fragments of plasmids pJP4 and pYG2 (from clone YG2) are shown in Table 2. The size of pYG2 is about 59 kb, as calculated from summing of the size of the restriction endonuclease fragments, whereas electron microscopic contour length measurement demonstrated its size to be about 93 kb. This plasmid when denatured and annealed shows a 24.5-kb stem with 1.3-kb single-stranded loop (Fig. 2*a*), suggesting the inverted duplication of a large segment of a region around *Eco*RI E fragment of pJP4. From the *Eco*RI digest of pYG2 (Fig. 1*c*, lane 6) and the hybridization data of individual pYG2 *Eco*RI fragments with pJP4 *Eco*RI fragments (Table 2), it is clear that *Eco*RI fragments C (11 kb), H (1.75 kb), and I (1.3 kb) of pJP4 have been deleted, resulting in the generation of pYG2. Restriction endonuclease analysis with *Bam*HI enzyme also revealed the deletion of 3.2-kb *Bam*HI B2 (denoted as B2 in Fig. 1*a*) fragment and duplication of 3.5-kb *Bam*HI B1 and 1.6-kb *Bam*HI B3 (denoted as B1 and B3 in Fig. 1*a*) fragments, thereby giving rise to a new 10.2-kb *Eco*RI fragment (pYG2 *Eco*RI C). This new 10.2-kb fragment co-migrates with *Eco*RI D fragment of pJP4 as a doublet in pYG2 (Fig. 1*c*, lane 6, fragment 3). Therefore, in plasmid pYG2, ≈17 kb of DNA from pJP4 has been deleted. Partial duplication of 2.8-kb *Eco*RI F fragment of pJP4 gave rise to *Eco*RI D fragment (5.5 kb) of pYG2.

Since plasmid pYG2 is 93 kb and 17 kb of pJP4 is deleted to give rise to pYG2, it is obvious that there is about 25 kb of additional DNA, which appears as inverted duplication of the segment comprising *Eco*RI B (15 kb), G (1.95 kb), F (2.8 kb), and part of *Eco*RI E (5.1 kb), which altogether comprise 24.85 kb, a good agreement between gel data and electron microscopic measurements. The *Eco*RI fragments A and D of pJP4 are not duplicated in pYG2 and are shown as a 43-kb single-stranded loop (Fig. 2*a*). To confirm these results,

Table 2. *Eco*RI fragment pattern, molecular size (kb), and hybridization of various fragments in plasmids pJP4 and pYG2

pJP4*		pYG2*		Hybridization profile with pJP4 fragments
*Eco*RI A	27.5	*Eco*RI A	27.0	*Eco*RI A
*Eco*RI B	15.0	*Eco*RI B	15.0	*Eco*RI B
*Eco*RI C	11.0	*Eco*RI C	10.2	*Eco*RI E, *Eco*RI D
*Eco*RI D	10.0	*Eco*RI D	5.5	*Eco*RI F
*Eco*RI E	8.36	*Eco*RI E	1.91	*Eco*RI G
*Eco*RI F	2.8			
*Eco*RI G	1.95			
*Eco*RI H	1.75			
*Eco*RI I	1.30			
	79.66 (85)†		59.61 (93)†	

The composition of five *Eco*RI fragments of pYG2 was derived from Southern blot analysis using isolated *Eco*RI fragments of pYG2 as probes by hybridizing various restriction fragments of plasmid pJP4 (see Fig. 1*c*, lane 8, for pJP4 and lane 6 for pYG2).

*The size of fragments was calculated in kb from agarose gel electrophoresis using phage λ DNA cut with *Hind*III as a molecular weight marker.

†Calculated from electron microscopic contour length measurement. The value under pYG2 agrees closely with a deletion of 17 kb of DNA from pJP4 followed by a duplication of a segment of about 25 kb.

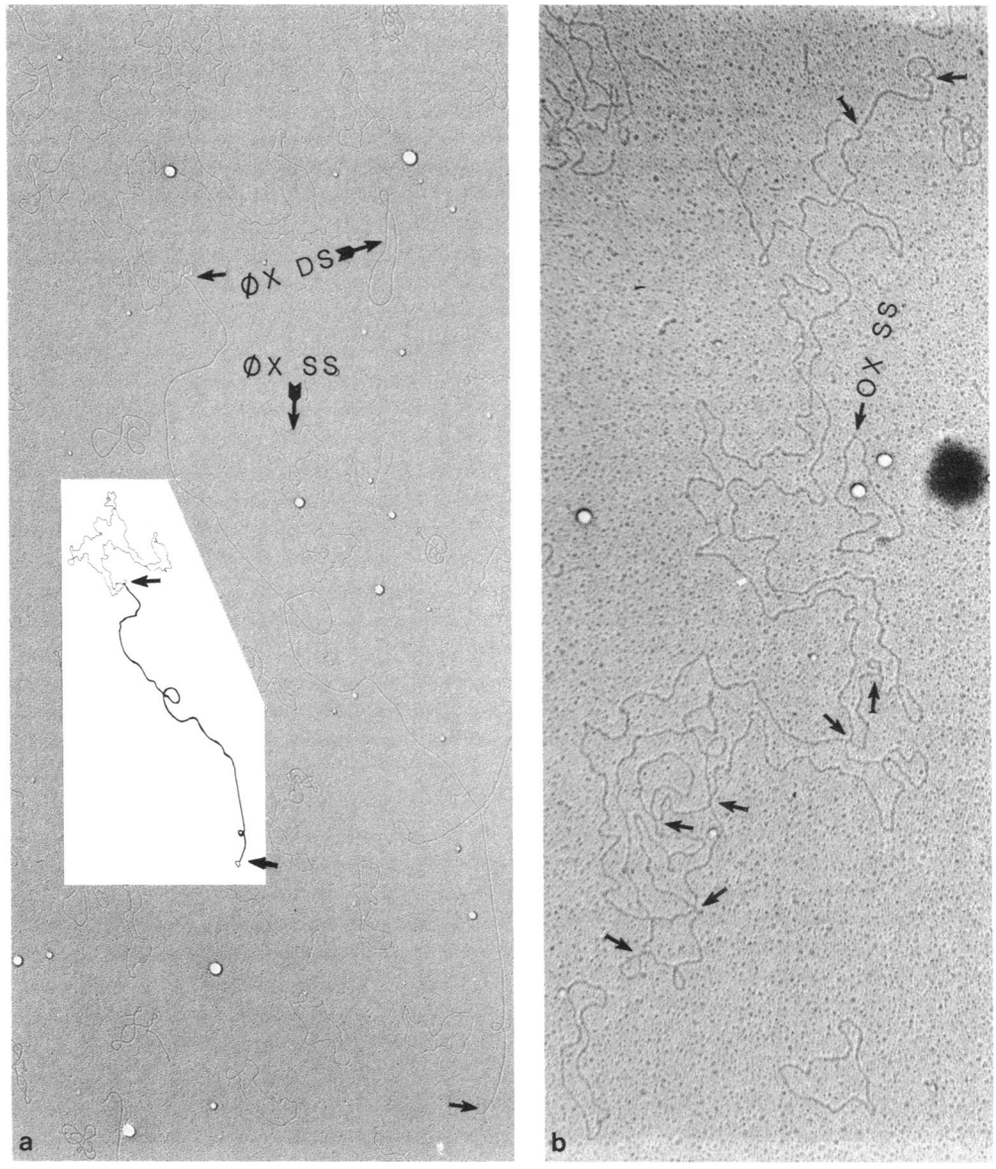

FIG. 2. (*a*) Electron micrograph of pYG2 DNA showing a 24.5-kb inverted duplication (arrow) with 43-kb single-stranded loop after denaturation and renaturation. A diagram of the molecule is also presented. ϕX174 double-stranded (ϕX DS) and single-stranded (ϕX SS) are also shown. (*b*) Electron micrograph of pJP4 DNA denatured and renatured showing a 1.8-kb stem and 1-kb loop (top arrows). The same molecule also shows several smaller inverted repeats with various loop sizes (marked by arrows).

*Eco*RI A–E fragments of pYG2 were purified and hybridized to various restriction enzyme digests of pJP4 and vice versa by Southern blot analysis. The results of these experiments are summarized in Table 2. *Eco*RI G fragment of pJP4 was found to undergo a very small deletion in pYG2 of a segment that is directly repeated in *Eco*RI fragments A, F, and G of pJP4, as evident from cross-hybridization in Southern blotting and the absence of stem–loop structure of expected size in homoduplex analysis (data not shown). This cross-hybridization was detected between *Eco*RI G fragment of pJP4 with *Eco*RI E fragment of pYG2. Similar rearrangements occurred in plasmid pJP4 when *A. eutrophus* JMP134 was successfully grown in minimal 3-chlorobenzoate liquid medium. Isolation of plasmid DNA showed a mixed profile on *Eco*RI digestion—i.e., the presence of both plasmid pJP4 and pYG2 forms (Fig. 1*c*, lane 7).

pJP4$^+$ transconjugants of *P. putida* or *P. cepacia* selected on *Pseudomonas* isolation agar plus merbromin grew extremely slowly on 3-chlorobenzoate, were Hg^r, and were always found to contain intact plasmid pJP4 (without rearrangements). No clone of *P. cepacia* harboring pJP4 was found to grow on minimal 3-chlorobenzoate liquid medium, presumably because of the lack of initial host enzymes specifying conversion of chlorobenzoate to chlorocatechol. Such enzymes appear to be coded by chromosomal genes in *P. putida* and *A. eutrophus*.

Structural Features of Plasmid pJP4. Unique DNA sequence rearrangements involving amplification, deletion–fusion, etc., in pJP4 resulting from selection on various growth substrates are likely to involve homologous DNA sequences in both direct and inverted orientation located on distinct points on the plasmid genome (8). The direct repeats were detected from Southern hybridization using a purified restriction fragment of the homologous plasmids, whereas inverted repeats were revealed as snap-back stem–loop structures in electron microscopic study.

Such studies revealed inverted repeats of sizes 1.8 kb to 50 base pairs with loop sizes from 0.5 kb to the ≈12-kb range and a few of these structures are shown in Fig. 2*b*. Most redundant sequences are present in *Eco*RI F fragment and show cross-hybridization with *Eco*RI fragments A and G and to B and C with greater mismatch. Approximate coordinates

of a few of these structures are known, whereas the position of most of them on the physical map of pJP4 is unknown. The role of such repeats as well as of the host recombination system in introducing deletions and other genetic rearrangements in pJP4 awaits further investigation.

DISCUSSION

There appears to be a common evolutionary emergence of the 3-chlorobenzoate degradative pathways encoded by plasmids pJP4 and pAC27, as indicated from the DNA·DNA hybridization study. Similarity of the 3-chlorobenzoate pathways encoded by the two plasmids (3) and mode of amplification of the structural genes specifying 3-chlorobenzoate degradation (that are necessary for high-level expression under selective pressure) through a gene dosage effect are all in agreement with this assumption.

The cloning data identify the location of at least three structural genes for pyrocatechase II, cycloisomerase II, and hydrolase II involved in 3-chlorobenzoate metabolism (3) on a 4.2-kb *Bgl* II E fragment of pAC27 and in the 10-kb *Bam*HI/*Eco*RI fragment of pJP4. These three enzymes are known to be essential for chlorocatechol degradation by the host cells. Our preliminary observations indicate that the first two enzymes of the 3-chlorobenzoate pathway—i.e., benzoate oxygenase and dihydrodihydroxybenzoate dehydrogenase—are not encoded by genes present on the *Bgl* II E fragment. The reason for the homology between the 8.36-kb *Eco*RI E fragment of pJP4 with the *Bgl* II E (4.2 kb) fragment of pAC27 is unclear. The pJP4 *Eco*RI E fragment, similar to the pAC27 *Bgl* II C fragment, is believed to harbor gene(s) necessary for efficient expression of the structural genes borne on pJP4 *Eco*RI B or pAC27 *Bgl* II E fragments. This was suggested from the experiment in which the plasmid pY43 containing *Eco*RI fragments EB, EE, and EF of pJP4 when mobilized to *P. putida* resulted in rapid 3-chlorobenzoate utilization phenotype, whereas many other combinations without the *Eco*RI E fragment failed to do so. Amplification of the cloned 4.2-kb *Bgl* II E of pAC27 takes place in the absence of the adjoining *Bgl* II C fragment. Similarly, amplification of *Eco*RI B fragment of pJP4 was not found in clone pYG1943. Interestingly, amplification of the cloned fragments in both pDC25 and pYG410 or pYG419 involves *Bgl* II and *Eco*RI restriction sites, respectively. This type of amplification is well documented in plasmid tetracycline resistance genes in several systems (9). At present it is not known whether this amplification involves the presence of direct repeats or the homology-dependent $recA^+$ system of the host cell. Absence of positive regulatory genes for elements, as reported in the case of the naphthalene operon genes of the NAH plasmid (5) and the *xylABC* and *xylDEFG* operons in the TOL plasmid (10), may result in a gene dosage effect through gene amplification in DC25 or YG410 and 419 under strong selection pressure.

It is interesting to note that although the structural genes are homologous in the two systems, their evolutionary divergence is evident from the mode of their expression. The 3-chlorobenzoate genes in plasmid pAC27 are readily expressed when transferred within *Pseudomonas* species. The same set of genes on pJP4 behaves like a cryptic operon that is activated occasionally through an unconventional mechanism of complex sequence rearrangements as is known in other cases such as insertion, deletion–fusion, or other types of illegitimate recombination (8).

The plasmid DNA from 3-chlorobenzoate-grown *Alcaligenes*, when analyzed under the electron microscope, showed the presence of three different classes of molecules based on size: 85 kb (probably parental pJP4), 93 kb (most likely pYG2), and a few molecules of size 100 kb or larger, which are most likely amplified intermediates before deletion–fusion. In denatured and fast renatured homoduplex molecules of such preparations, the three classes were well distinguished by their characteristic snap-back structures: 1.8-kb stem, 1-kb loop of parental pJP4, 24.5-kb stem, 1.3-kb loop of pYG2 form, and few very large complex molecules with many "underwound loops." This indicates that duplication and deletion occur in separate steps, probably duplication preceding deletion. The potential plasticity of the plasmid pJP4 in its catabolic versatility is indicated in its ability to degrade 2,4-D, 3-chlorobenzoate, and 4-chlorophenoxyacetic acid (3). Since 2,4-D degradation seems to occur through the chlorocatechol intermediate in pJP4, the chlorocatechol-degrading genes can be recruited for 3-chlorobenzoate degradation in the presence of chromosomal benzoate oxygenase and dihydrodihydroxybenzoate dehydrogenase genes, whose gene products may have a broad specificity to convert 3-chlorobenzoate to 3-chlorocatechol. At present, no clear-cut rationale can be put forward to explain the mechanism of formation of the deletions and the selective amplification of the structural genes as well as the functional significance of such DNA sequence rearrangements in the expression of 3-chlorobenzoate phenotype encoded by pJP4.

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