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Recombination of a 3-Chlorobenzoate Catabolic Plasmid from *Alcaligenes eutrophus* NH9 Mediated by Direct Repeat Elements

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Alcaligenes eutrophus **NH9 was isolated from soil. This strain can utilize 3-chlorobenzoate (3-CB) as a sole source of carbon and energy. Most of the 3-CB-negative segregants had lost one of the plasmids present in the parent strain. The genes for catabolism of 3-CB were located within a 9.2-kb *Sac*I fragment of this plasmid (pENH91). The genes were found to hybridize with genes for components of the modified *ortho* cleavage pathway from *Pseudomonas putida*. In one of the 3-CB-negative segregants, the plasmid had undergone the deletion of a segment with a size of about 12.5 kb that covered the catabolic genes. The deletion event seemed to be the result of reciprocal recombination between two highly homologous sequences with sizes of 2.5 kb that were present as a direct repeat at the two ends of the region that included the catabolic genes. Nucleotide sequence analysis of homologous fragments revealed a structure that resembled an insertion sequence and relatedness to IS*21*. During repeated subculturing of NH9 on liquid media with 3-CB, the culture was taken over by a derivative strain (designated NH9A) in which the degradative plasmid carried a duplicate copy of the 12.5-kb region that contained the catabolic genes. The duplication of these genes seemed again to have been mediated by recombination between the direct repeat sequences.**

Chlorinated aromatic compounds pose one of the most serious contemporary environmental problems worldwide because they have been used in large quantities as herbicides, pesticides, and solvents. Although some of these compounds persist for long periods in the environment, others are degraded by microorganisms in soil and water (1). It is important to study the biochemical and genetic basis of microbial degradation to find ways to improve the degradative ability of microorganisms as well as to understand the evolution of the genes for degradative enzymes. In the oxidative bacterial degradation of chlorinated aromatics, reactions known collectively as the modified *ortho* cleavage pathway play a pivotal role (41). 3-Chlorobenzoate (3-CB), 2,4-dichlorophenoxyacetic acid, and 1,2,4-trichlorobenzene are converted to the corresponding chlorinated catechols by *Pseudomonas putida* (10), *Alcaligenes eutrophus* JMP134 (15), and *Pseudomonas* sp. strain P51 (43), respectively. These chlorinated catechols are then metabolized by the enzymes of the modified *ortho* cleavage pathway, the genes for which are clustered in similar operons carried by plasmids pAC27 in *P. putida* AC866 (17) (or the highly homologous plasmid pWR1 in *Pseudomonas* sp. strain B13 [8]), pJP4 in *A. eutrophus* JMP134 (15, 31), and pP51 in *Pseudomonas* sp. P51 (42, 43). The homology of DNA sequences and deduced amino acid sequences and the structural similarities among the three operons for degradation of chlorocatechols imply that they are derived from a common ancestor (42).

Genetic rearrangements and transposability of the degradative genes have been another focus of interest. There are a few reports of genetic recombination related to the degradation of 3-CB. Amplification and deamplification of the 4.3-kb fragment that contains the structural genes for degradation of chlorocatechols were demonstrated in *Pseudomonas* sp. strain B13 (33). Amplification of cloned fragments containing the *clc* structural genes from plasmid pAC27 and pJP4 was reported (20). Inverted duplication of catabolic genes on pJP4 and preexisting tandem duplication of catabolic genes on a related plasmid, pJP2, were also described (19, 20). Although each of these events seemed to have been mediated by homologous recombination, the nucleotide sequences responsible for the recombination have not been determined in any of these cases.

A few reports of transposons that carry the genes for degradation of chlorinated aromatics have appeared in the literature. The *tcbAB* gene cluster of plasmid pP51 encoding enzymes that convert 1,2,4-trichlorobenzene to 3,4,6-trichlorocatechol is located on a composite transposon with two slightly different insertion sequences in an inverted orientation at its ends (44). A 17-kb segment of plasmid pBRC60, specifying enzymes for the degradation of 3-CB in *A. eutrophus* BR60, was found to be a composite transposon flanked by directly repeated 3.2-kb segments of class II insertion sequences (28). Strain BR60 of *A. eutrophus* is unique in that it degrades 3-CB by way of protocatechuate *meta* ring fission, and its catabolic genes are different from the degradative genes of plasmid pAC27 (29, 46). Although plasmids that carry genes for the modified *ortho* cleavage pathway have been found in widely separated places in the world, there have been no reports of a transposon that carries genes for this pathway.

In the present report, a plasmid that encodes 3-CB-degrading enzymes from a strain of *A. eutrophus* is described. The genes for the catabolic enzymes, which hybridized with genes for enzymes of the modified *ortho* cleavage pathway of *P. putida* AC866 (7, 18), were flanked by directly oriented homologous elements with sizes of 2.5 kb that were related to IS*21* (34). To our knowledge, this is the first report of a set of insertion sequence-like elements that were involved in recombination events, including both deletion and amplification of genes for the modified *ortho* cleavage pathway. We demonstrated the occurrence of recombination between the two insertion sequence-like elements by isolating plasmids and comparing their restriction and hybridization patterns. Although transposition was not detected, the composite transposon-like

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TABLE 1. List of strains, plasmids, and phages

Strain, plasmid, or phage	Relevant characteristics	Source or reference
E. coli		
B378	DH1(RP4)Ap^r Km^r Tc^r *tra*$^+$	37
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	47
XLI-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*$^-$ F′[*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(Tet^r)]	5
S17-1	C600::RP4 2-(Tc::Mu)(Km::Tn*7*) *thi pro hsdR hsdM*$^+$ *recA*	39
Plasmids and phages		
pPSA842	Ap^r Sm^r Tc^r *mob*$^+$ *tra* λ *cos*	37
pKT230	Km^r Sm^r *mob*$^+$	3
pUC18,19	Ap^r *lacZ*α$^+$	47
M13mp18,19	*lacZ*α$^+$	30
pDC100	pMMB22 carrying the 4.3-kb *Bgl*II E fragment of pAC27; Ap^r	18
A. eutrophus		
NH9	pENH91, 3-CB$^+$	This study
NH9D	3-CB$^-$	This study
NH9d5	pENH91d5, 3-CB$^-$	This study
NH9A	pENH91A, 3-CB$^+$	This study

structure suggests that the genes for chlorocatechol-degrading enzymes might have been spread by transposition as well as by transmissible plasmids.

MATERIALS AND METHODS

Bacteria, plasmids, phages, and growth conditions. The bacterial strains, plasmids, and phages used in this study are listed in Table 1. All strains of *Escherichia coli* were grown in Luria-Bertani medium (35) at 37°C. Strains of *A. eutrophus* were grown at 30°C. Strain NH9 was routinely maintained on basal salts medium [per liter: $(NH_4)_2SO_4$, 1.1 g; K_2HPO_4, 2.29 g; KH_2PO_4, 0.9 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $MnSO_4 \cdot 4{\sim}6H_2O$, 0.025 g; $FeSO_4 \cdot 7H_2O$, 0.005 g; L-ascorbic acid, 0.005 g] that contained 0.1% 3-CB (final pH, 7.0). Luria-Bertani medium, nutrient broth medium (per liter: beef extract, 5 g; peptone, 5 g; NaCl, 2.5 g; [pH unadjusted]), and glucose-yeast extract (GY) medium [per liter: glucose, 1 g; yeast extract, 1 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $Fe_2(SO_4)_3 \cdot 7H_2O$, trace (pH 6.8)] were used for strains of *A. eutrophus*. M9 minimal medium (35) with 0.2% mannitol, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 1 mM thiamine hydrochloride was used as the minimal medium for the selection of recipient strains in conjugations with transfers of plasmids from *A. eutrophus* to *E. coli*. Antibiotics were incorporated into media at the following final concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; streptomycin, 25 μg/ml; and tetracycline, 15 μg/ml.

Isolation of DNA. Large plasmids from *A. eutrophus* were prepared by the method of Casse et al. (6), which was followed by cesium chloride-ethidium bromide centrifugation. Recombinant plasmids from *E. coli* were prepared either by the alkaline lysis method of Kieser (26) or by the "small-scale preparations" method described by Sambrook et al. (35). Total DNA from *A. eutrophus* was prepared by a method described by Schmidt et al. (38).

Manipulation of DNA. Digestion with restriction endonucleases, DNA ligations, and transformation of *E. coli* with plasmid DNA were performed basically by methods described by Sambrook et al. (35). Plasmids pUC18 and pUC19 were used routinely for subcloning of DNA fragments. Transformation of *E. coli* JM109 or XLI-Blue was carried out either by the method of Hanahan (21) or by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.) (40).

Conjugation and incompatibility test. Conjugation was performed by a method described by Franklin (16). The conclusion of incompatibility tests was based on the observation of the appearance of the transconjugants of *A. eutrophus* that retained the ability to use 3-CB and exhibited the phenotype of antibiotic resistance of the introduced plasmid.

Southern hybridization. Southern blotting to nylon membranes (Hybond-N; Amersham International plc, Amersham, United Kingdom) was performed by the standard procedure (35) or with a vacuum-blotting apparatus (VacuGene; Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). DNA restriction fragments were labeled by nick translation or random priming (35) with [α-^{32}P]dCTP (Amersham). Hybridizations were performed overnight at 42°C by a method described elsewhere (35). The hybridization solution consisted of (per 10 ml) 5.08 ml of formamide, 0.50 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (0.5 M, pH 7.5), 2.25 ml of a solution of nonhomologous DNA in water (1 mg/ml), 0.10 ml of 100× Denhardt's solution, 0.56 ml of distilled water, and 1.50 ml of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Washing was performed under high-stringency conditions: twice for 15 min with 2× SSC at 65°C, once for 30 min with 2× SSC and 0.1% sodium dodecyl sulfate at 65°C, and then once for 10 min with 0.1× SSC at 65°C.

Sequencing. The 3.3-kb *Sal*I-*Sph*I fragment containing DR2 was subcloned into M13mp18 and M13mp19. For sequencing, nested deletions were introduced in both directions and clones with inserts of appropriate lengths were selected. Sequencing was carried out by the dideoxy method (36) either with a Sequenase version 2.0 kit (United States Biochemical Corporation, Cleveland, Ohio) and [α-^{35}S]dCTP (Amersham) or with a Dye Primer Cycle Sequencing kit and an automated sequencer (model 373A; Perkin-Elmer, Norwalk, Conn.).

Nucleotide sequence accession number. The nucleotide and amino acid sequence data reported in this study have been submitted to the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession number D64144.

RESULTS

***A. eutrophus* NH9 and its plasmids.** *A. eutrophus* NH9 was isolated from a soil sample at our institute under conditions in which 3-CB was the sole source of carbon and energy, and it was subsequently subcultured on 3-CB-containing mineral salts agar plates. Taxonomic identification was carried out by Katoh (25).

Mutants of NH9 unable to utilize 3-CB (3-CB$^-$) arose spontaneously and at high frequency (>10%) after repeated subculture (five times) on GY liquid medium. It appeared, therefore, that some of the functions necessary for degradation of 3-CB were plasmid encoded.

With a slight modification to the method of Casse et al. (6), we showed that NH9 harbored two large plasmids (Fig. 1, lane 1). The yield of the larger plasmid (designated pENH92) was much lower than that of the smaller one (pENH91). Many of the 3-CB$^-$ mutants obtained as independent colonies after subculture on GY medium had been cured of pENH91 (Fig. 1, lanes 2 to 5, 7, and 8). Although pENH91 appeared to be retained in one of the 3-CB$^-$ mutants (Fig. 1, lane 6 [designated NH9d5]), the plasmid seemed to be smaller than the original one. These results suggested that some of the genes essential for the degradation of 3-CB were carried by pENH91.

pENH91 was transferred from wild-type NH9 cells to spontaneous 3-CB$^-$ Sm^r cells by conjugation. Transfer into the recipient strain occurred at a frequency of 10^{-4} per donor cell.

pENH91 was tested for its incompatibility with pKT230, pPSA842 (IncQ), and RP4 (IncP1). Plasmids pKT230 and pPSA842 were mobilized from *E. coli* S17-1 to NH9, and RP4

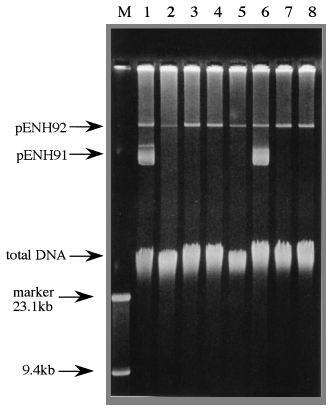

FIG. 1. Agarose gel electrophoresis of the plasmids from strain NH9 and from 3-CB$^-$ segregants (0.7% gel and Tris-borate-EDTA buffer). Lanes: M, lambda DNA digested with *Hin*dIII; 1, *A. eutrophus* NH9; 2 to 8, 3-CB$^-$ segregants of *A. eutrophus* NH9.

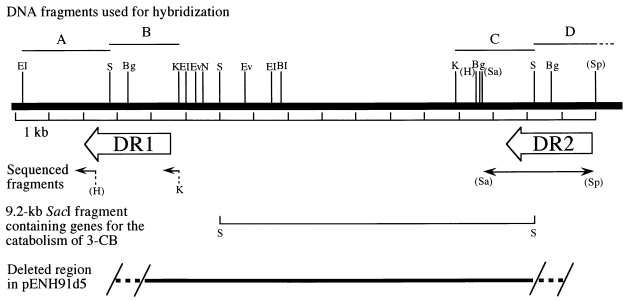

FIG. 2. Physical map of the region that includes the catabolic genes. Horizontal lines above the linear restriction map show the fragments (A and B) used as probes in hybridization experiments and the hybridized fragments (C and D), respectively. The open arrows (DR1 and DR2) correspond to the insertion sequence-like elements and the direction of the three ORFs in DR2. The regular arrows indicate the directions of sequencing and the lengths of sequences determined. Under the arrows, the 9.2-kb *Sac*I fragment containing genes for the catabolism of 3-CB is shown. The thick solid line at the bottom shows the deleted region in pENH91d5. Restriction sites are abbreviated as follows: BI, *Bam*HI; Bg, *Bgl*II; EI, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; N, *Nhe*I; S, *Sac*I; Sa, *Sal*I; and Sp, *Sph*I. Restriction sites in parentheses are those determined only for the subcloning of related fragments, and thus, other sites for the enzymes on the linear map were not determined.

was transferred from *E. coli* B378 to NH9. The test was based on the observation of the appearance of transconjugants of *A. eutrophus*, which can grow on 3-CB agar plates supplemented with kanamycin (for pKT230 and RP4) or ampicillin (for pPSA842). Transconjugants resistant to kanamycin and ampicillin were obtained after the introduction of pKT230 and pPSA842, respectively. The presence of the two plasmids (pENH91 plus either pKT230 or pPSA842) was verified after the reintroduction of the plasmids into *E. coli*. The plasmids were identified by isolation and subsequent restriction analysis. Thus, pENH91 was found to be compatible with pKT230 and pPSA842. But we found that pENH91 could not be maintained with RP4 in a stable manner. Thus, pENH91 was considered to be incompatible with RP4 and therefore is likely an IncP group plasmid.

Hybridization with catabolic genes from *P. putida*. Cloning experiments showed that genes essential for the catabolism of 3-CB were located within a 9.2-kb *Sac*I fragment of plasmid pENH91 (Fig. 2). (Details of cloning will be described elsewhere.)

To identify the determinants responsible for the catabolism of 3-CB on pENH91, hybridization experiments were conducted with the chlorocatechol catabolic genes of pAC27 (7), which had been originally isolated from *P. putida* AC866, as a probe. The 4.3-kb fragment containing the *clcABD* genes of pAC27, isolated from an *Eco*RI digest of pDC100, hybridized to the 5.8-kb *Bam*HI-*Bgl*II fragment from within the 9.2-kb *Sac*I fragment (data not shown). Therefore, the catabolic genes on pENH91 appeared to encode enzymes of the modified *ortho* pathway.

Deletion of the catabolic genes from pENH91. A 12.5-kb region containing the 9.2-kb *Sac*I fragment (Fig. 2) was found to have been deleted from the plasmid harbored by one of the 3-CB$^-$ segregants (Fig. 1, lane 6 [designated pENH91d5]). (For a result of hybridization experiments, see Fig. 7a and b, lanes 3.) Two fragments of pENH91 which encompassed either the left or right boundary region of the deleted segment showed strong homology to each other in a hybridization experiment (Fig. 3; see fragments B and D in Fig. 2). A 2.5-kb *Eco*RI-*Sac*I fragment (fragment A in Fig. 2) hybridized with a 2.3-kb *Kpn*I-*Sac*I fragment (fragment C) (data not shown). These results indicated that the two homologous fragments were in the same orientation and suggested that the deletion event had been due to homologous recombination between the two elements. On the restriction map (Fig. 2), the left homologous sequence was designated as DR1 and the right homologous sequence was designated as DR2. When the 2.0-kb *Sac*I-*Kpn*I fragment (fragment B in Fig. 2) isolated from pENH91 was used as a probe against pENH91d5, only one hybridized band for pENH91d5 instead of two bands for pENH91 appeared (data not shown). Further restriction analysis with pENH91d5 showed that this plasmid retained one sequence of the direct repeat elements and thus confirmed that the deletion of the catabolic region was due to homologous recombination between the two elements.

Sequence analysis of the direct repeats. A 3.3-kb *Sal*I-*Sph*I fragment containing DR2 was subcloned into M13 vectors and sequenced. The boundary regions of DR1 and DR2 were determined by hybridization, and fragments that included either of the two termini of DR1 were also sequenced (Fig. 2). DR1 and DR2 contained the same sets of 16-bp inverted repeats at their ends, with two mismatches (Fig. 4). There were no target site duplications flanking the extreme ends of the 15-kb region containing the two elements or the termini of either DR1 or DR2 (Fig. 4). The nucleotide sequences of the left and right end regions of DR1 were compared with those of the corresponding end regions of DR2, and the sequences were found to be identical (left end, 234 bp; right end, 296 bp). The restriction sites for *Sac*I, *Bgl*II, and *Hin*dIII were retained by the two direct repeats. These observations, together with the data from the hybridization analysis, suggested that the two members of the pair of direct repeat sequences (DR1 and DR2) might be identical.

ORFs of DR2. DR2, including the inverted repeats, was 2,521 bp long (Fig. 5). Four long open reading frames (ORFs)

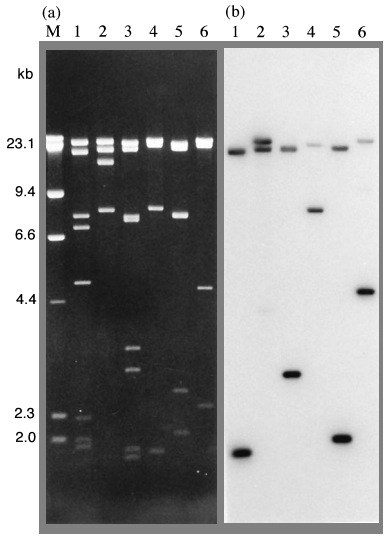

FIG. 3. Southern hybridization of fragments of pENH91 with the 2-kb *Sac*I-*Kpn*I fragment, designated B, as the labeled probe. (a) Agarose gel electrophoresis of fragments of pENH91 (0.7% gel and Tris-borate-EDTA buffer). Lanes: M, lambda DNA digested with *Hin*dIII as size standards; 1 to 6, pENH91 DNA digested with restriction enzymes (1, *Sac*I and *Kpn*I; 2, *Kpn*I; 3, *Sac*I and *Bam*HI; 4, *Bam*HI; 5, *Sac*I and *Eco*RI; 6, *Eco*RI). (b) Southern hybridization of a filter prepared from the gel in panel a and probed with the 2-kb *Sac*I-*Kpn*I fragment B of pENH91 located on the left part of the linear map shown in Fig. 2 (the lanes correspond to those in panel a). In each lane, there are two hybridized bands. One contains fragment B used as the probe, and the other contains fragment D located on the far right of the linear map in Fig. 2.

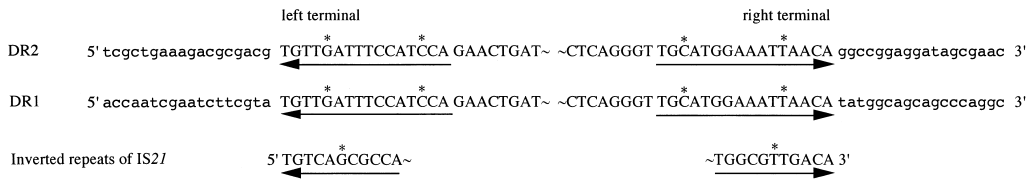

FIG. 4. Terminal regions of direct repeats of pENH91 and inverted repeats of IS*21*. The underlined nucleotide sequences are inverted repeats, and the arrows indicate their orientations. Nucleotides of the insertion sequence-like elements and IS*21* (34) are shown in uppercase letters. Nucleotides outside of the insertion sequence-like elements are shown in lowercase letters. Note that here the left and right designations refer to the directions of the ORFs found in DR2, as discussed in the text, and thus, they are the reverse of the directions shown in Fig. 2. Nucleotides that are mismatched in the left and right inverted repeats in each element are marked by asterisks.

were found on one DNA strand of DR2 (Fig. 5 and 6). The amino acid sequences predicted from three ORFs exhibited homology to those of IS*21* (34). ORFA1 exhibited homology to the amino-terminal half of IstA of IS*21*, while ORFA2 exhibited homology to the carboxyl-terminal half of IstA (Fig. 6). ORFB exhibited homology to IstB of IS*21*. ORFu (in frame with ORFA1) had no apparent relationship to any of the amino acid sequences in the major databases.

There were two possible initiation codons for ORFA1. This ORF had the capacity to encode a protein of 251 (or, alternatively, 248) amino acids, if translation was initiated at the GTG codon at position 106 (or position 115) (Fig. 5). Each of these codons was preceded by a potential ribosome-binding site (SD1 or SD2). ORFA2 consisted of 338 codons. There was no possible ribosome-binding site for ORFA2. ORFB encoded a putative protein of 264 amino acids, if the ATG codon of position 1653 (Fig. 5) was used as an initiation codon. This codon was preceded by a possible ribosome-binding site (SD3).

The amino acid sequences and overall structural similarities (Fig. 6) suggested an evolutionary relationship between DR2 and IS*21*.

The G+C content was 63.6% for DR2 and 52.3% for IS*21*. The G+C content of DR2 was slightly lower than the G+C content of the total genomic DNA of *A. eutrophus* (66.3 to 66.8%) (12). The G+C contents of the third positions in the codons used for ORFA1, ORFA2, ORFB, and ORFu in DR2 were 82.9, 79.6, 80.7, and 41.4%, respectively. Those for IstA and IstB were 62.6 and 60.9%, respectively. By contrast to the ORFs of IS*21*, the codon usage in ORFA1, ORFA2, and ORFB was highly biased in favor of G or C in the third position. This phenomenon has also been observed in other genes from *A. eutrophus* (2, 23, 32). The low G+C content of the third positions of the codons of ORFu suggests that this ORF is nonfunctional in *A. eutrophus.*

Duplication of the catabolic genes. After successive transfers of NH9 to fresh 3-CB-containing liquid medium at approximately 2-week intervals for a year, the cells in the culture were compared with NH9 by hybridization experiments with both plasmid DNAs and total DNAs to examine whether any recombination events had occurred. The cells and the catabolic plasmid from the 1-year-old subculture were designated strain NH9A and pENH91A, respectively.

The 5.8-kb *Bam*HI-*Bgl*II fragment of the cloned 9.2-kb *Sac*I fragment was labeled and used as a probe to examine *Eco*RI digests of plasmid DNAs and total DNAs from the two strains (Fig. 7). In the *Eco*RI digest of pENH91, a 40-kb *Eco*RI fragment containing the probe region was found to have hybridized (Fig. 7, lane 1). In the *Eco*RI digest of pENH91A, an additional 10-kb fragment appeared, and this novel fragment also hybridized strongly with the probe (Fig. 7, lane 2). Hybridization with *Eco*RI digests of total cellular DNA from the two strains gave the same patterns as those of the respective plasmid DNAs (Fig. 7, lanes 4 and 5). These results suggested that the novel 10-kb *Eco*RI fragment on pENH91A also contained the catabolic genes.

Several restriction profiles of pENH91 and pENH91A were compared. One *Nhe*I site, one *Bam*HI site, two *Eco*RV sites, and two *Eco*RI sites were present within the intervening region between DR1 and DR2 on pENH91, but there were no sites for any of these four restriction endonucleases in the direct repeat sequences (Fig. 2). On the contrary, *Bgl*II sites were found in the direct repeats of pENH91 as well as within the intervening region. All the fragments that were present in the digests of pENH91 were observed in the corresponding restriction profile of pENH91A. In addition, restriction profiles of pENH91A with *Nhe*I, *Bam*HI, *Eco*RV, or *Eco*RI gave an additional fragment (12.5 kb, 12.5 kb, 11 kb, and 10 kb, respectively). The profiles of *Bgl*II digests of the two plasmids were identical (data not shown).

A 1.0-kb *Hin*dIII-*Bgl*II fragment of DR2, when used as a probe, hybridized with two fragments that contained either DR2 or DR1 in each digest and with the additional fragments of pENH91A listed above (data not shown). The intensities of the three hybridization bands in each of the digests of pENH91A with *Nhe*I, *Bam*HI, *Eco*RV, or *Eco*RI were nearly equal. This result suggested that the molar amount of each of the three fragments in pENH91A was equal. Conversely, the hybridization patterns were the same for the two plasmids in the *Bgl*II digests.

When the 5.8-kb *Bam*HI-*Bgl*II fragment of the cloned 9.2-kb *Sac*I fragment was used as a probe, it hybridized with the novel fragments that appeared in the digests of pENH91A as well as with the fragments that initially contained the 5.8-kb region. Hybridization patterns with the *Bgl*II digests of the two plasmids were the same; this probe hybridized only with the 10.4-kb fragment that included the 5.8-kb region.

These results indicated that the novel fragment contained both the region that encoded the catabolic enzymes and the direct repeat and that there was a doublet band(s) in the electrophoretic profile of the *Bgl*II digest of pENH91A. If the novel fragment had been located at a distant site on the plasmid, for example, by transposition, it would have altered the sizes of the original fragments of pENH91. Because all the restriction fragments of pENH91 were retained in pENH91A, it was clear that the novel fragment was located close to the original region.

The recombination event must have occurred with one of the following two patterns: (i) -DR-CB-DR-CB-DR-, by homologous recombination between the direct repeats; or (ii) -DR-CB-DR-DR-CB-DR-, by replicative transposition (DR and CB indicate a direct repeat element and the intervening region of 3-CB-catabolic genes, respectively).

The length (12.5 kb) of the novel fragments generated by

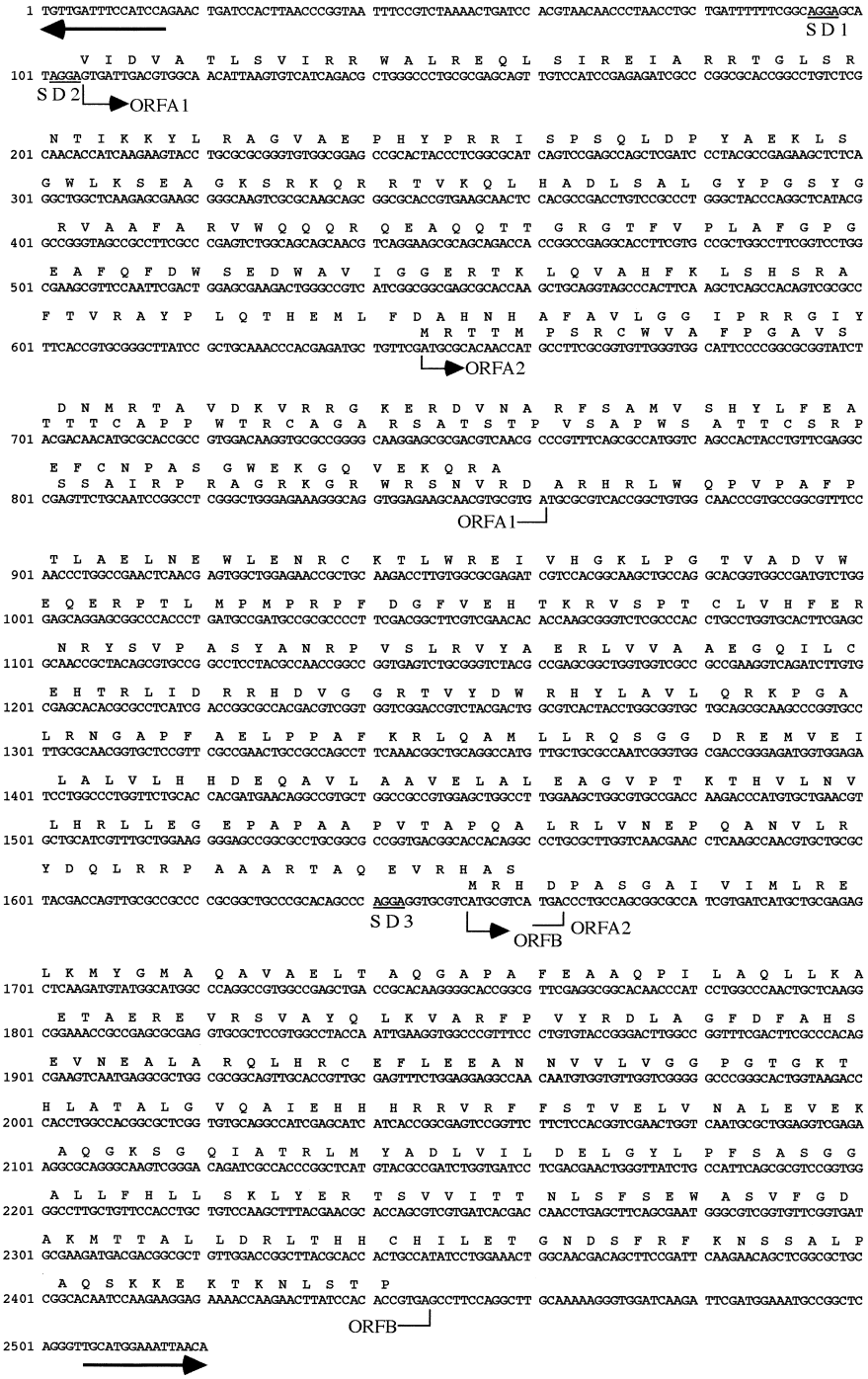

FIG. 5. Nucleotide sequence of DR2. Thick arrows show inverted repeats. ORFA1, -A2, and -B are indicated. The predicted amino acid sequences of ORFA1, -A2, and -B are shown above the first nucleotide of each codon. Shine-Dalgarno sequences are indicated as SD1 to -3.

digestion with either *Nhe*I or *Bam*HI was equal to the sum of the lengths of the intervening region (10 kb) and a direct repeat (2.5 kb). This result is in accord with pattern i. In addition, the identical profiles obtained with the *Bgl*II digests of the two plasmids allow us to exclude pattern ii. If pattern ii were to describe what had occurred, the *Bgl*II digest of pENH91A should have given an additional fragment of (at least) 2.5 kb, derived from the junctional region (-DR-DR-).

The lengths of the novel fragments generated by the four enzymes other than *Bgl*II corresponded to the lengths of fragments that would be produced by tandem duplication and not by inverted duplication.

These considerations confirmed that tandem duplication of the 12.5-kb region that contained the catabolic genes took place via reciprocal recombination between the direct repeats, as illustrated in Fig. 8.

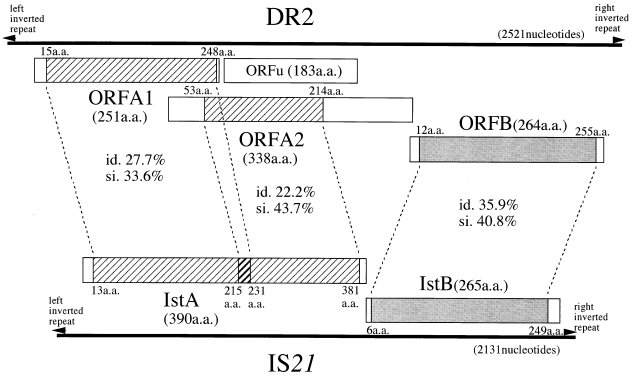

FIG. 6. Schematic representation of the ORFs of DR2 and IS*21* (34). Hatched and shaded areas indicate the regions that showed amino acid (a.a.) sequence homology. Percentages of identical amino acids (id.) and similar amino acids (si.) are indicated.

DISCUSSION

Plasmid pENH91 from *A. eutrophus* NH9 that carries genes for catabolism of 3-CB was characterized in this study.

The restriction pattern of pENH91 was different from those of other plasmids with 3-CB-degradative genes (9, 11). NH9 could not grow on 2,4-dichlorophenoxyacetic acid, and the restriction profile of pENH91 was different from those of 2,4-dichlorophenoxyacetic acid-degrading plasmids (4, 14, 27). Therefore, we concluded that pENH91 was a novel 3-CB-degradative plasmid.

The homologies and the overall structural similarity between DR2 and IS*21* indicated an evolutionary relationship.

Although the terminal inverted repeats of DR2 and IS*21* (Fig. 4) differed in length, there was some similarity between the nucleotide sequences. The inverted repeat of DR2 was TGT-N_{11}-CA, and that of IS*21* was TGT-N_6-CA (N = nucleotide). It is not known whether these apparently conserved nucleotides are important for the function of the insertion sequence (or insertion sequence-like) elements.

The entire 15-kb region containing DR1, DR2, and the catabolic genes exhibited a structure of a composite transposon, although there were not target site duplications. Tn*5271* is a well-described transposon that carries 3-CB-degradative genes (28, 29). The transposon-like structure on pENH91 is clearly different from that of Tn*5271*, as follows. (i) The insertion sequence-like elements on pENH91 are related to IS*21*, whereas IS*1071* of Tn*5271* belongs to the Tn*3* family of transposable elements. (ii) The catabolic genes of pENH91 appear to be genes for enzymes of the modified *ortho* cleavage pathway, whereas Tn*5271* carries genes for chlorobenzoate-3,4-dioxygenase. Preliminary sequencing data for the 9.2-kb *Sac*I fragment of pENH91 revealed a high degree of homology with the *tcbCDEF* genes of the 1,2,4-trichlorobenzene-degrading bacterium, *Pseudomonas* sp. strain P51 (42). The structures of the genes for catabolic enzymes encoded by pENH91 will be published elsewhere.

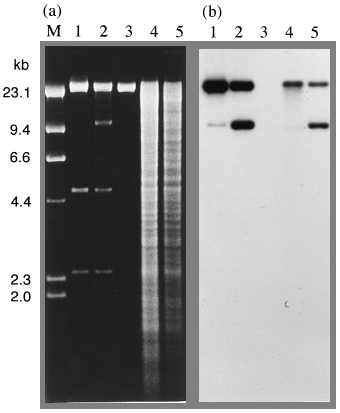

FIG. 7. Southern hybridization of restriction digests of plasmid DNA and total DNA from NH9 and derivative strains, with the 5.8-kb *Bam*HI-*Bgl*II fragment being used as the labeled probe. (a) Agarose gel electrophoresis (0.7% gel and Tris-borate-EDTA buffer) of *Eco*RI digests of samples of DNA. Lanes: 1, pENH91; 2, pENH91A; 3, pENH91d5; 4, total DNA from NH9; 5, total DNA from NH9A; M, fragments of lambda DNA digested with *Hin*dIII as size standards. (b) Autoradiogram of the corresponding Southern blot after hybridization with the 5.8-kb *Bam*HI-*Bgl*II fragment as the labeled probe. The lanes correspond to those in panel a.

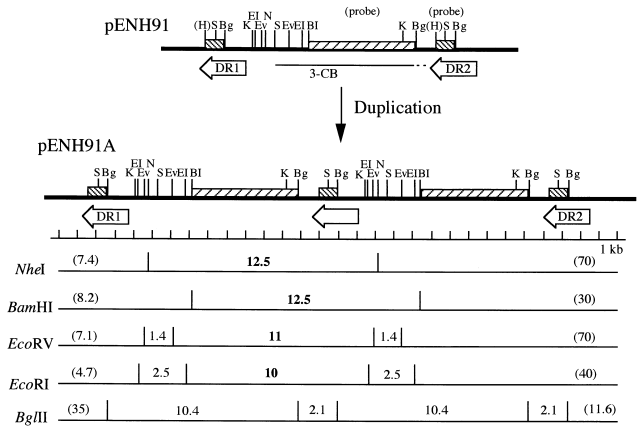

FIG. 8. Restriction map of the duplicated region of pENH91A. Hatched areas indicate the fragments that were used as probes in hybridization experiments and the corresponding hybridized regions. The numbers on the map for each restriction enzyme are the lengths of the fragments in kilobases. The fragments with numbers in bold type are the fragments that were not seen in the restriction profiles of pENH91. The fragments with numbers in parentheses are those whose entire lengths are not included in this figure. The abbreviations for the restriction sites are the same as those in the legend to Fig. 2.

Because pENH91 was found not to coexist stably with RP4, we introduced RP4 into NH9 by conjugation, expecting to obtain isolates in which the catabolic genes were transposed onto either RP4 or the chromosome of NH9. Although a few colonies appeared on plates prepared with 3-CB and kanamycin, none of these isolates was stable and no evidence of transposition has yet been obtained. Thus, the transposability of the 15-kb region remains to be demonstrated.

The structure of the 15-kb region might indicate that this region had been transposed into a region of a plasmid that was an antecedent of pENH91. But this region was not flanked by duplicated sequences of the target site, which was supposed to be generated by transposition. Some explanations are possible for this structure. (i) There could be an integration of an antecedent of pENH91 with one copy of an insertion sequence or insertion sequence-like element into a target site flanking chromosomal 3-CB genes by replicative transposition, followed by intramolecular transposition of the element into a site beyond the 3-CB genes and deletion. This mechanism has been proposed for the mobilization of catabolic genes into plasmids (45) and accounts for the lack of target-site duplications. (ii) Insertion of an insertion sequence or insertion sequence-like element proximal to the 3-CB genes of an antecedent of pENH91 that had originally contained the 3-CB genes, fol-

lowed by duplication of the element, could take place. This case has been observed for the 2,4,5-trichlorophenoxyacetic acid genes and associated insertion sequences (22). This mechanism can account for the observed 15-kb structure of pENH91 if the transposition of the element did not generate target-site duplication, as was found for a certain kind of insertion sequence element (13, 24).

Duplication and deletion of the degradative genes were demonstrated to be the consequences of recombination between the two insertion sequence-like elements.

There have been a few reports of the amplification of genes for 3-CB-degrading enzymes. The nucleotide sequences involved in these recombination events have not been reported. Amplification of the 4.3-kb region in *Pseudomonas* sp. strain B13 seemed to be necessary to avoid the accumulation of intermediate products of the degradation of 3-CB (33). Similar amplifications of a cloned 4.2-kb fragment from pAC27 and a 15-kb fragment from pJP4 were also ascribed to the necessity for high gene dosage, for example, in order to compensate for the absence of a positive regulatory element (20). In the case of the inverted duplication on pJP4 and the preexisting tandem duplication on pJP2, the advantage was attributed to mutation of the duplicated copy, with the resultant acquisition of new catabolic phenotypes, rather than to a gene dosage effect (19).

No major differences between NH9 and NH9A have yet been observed when features of their growth in 3-CB-containing liquid medium or their tolerances of 3-CB are compared. In Fig. 7b (lanes 1 and 4) (plasmid DNA and total DNA of NH9, respectively), very weakly hybridized bands can be seen at the same size as the extra fragment of the duplicated plasmid of NH9A. This suggested that cultures of NH9 would contain NH9A at a low ratio. However, the fact that NH9A became dominant after 1 year of successive subculturing indicates that duplicated catabolic genes provide cells with an advantage under our laboratory conditions. The functional significance of gene duplication on pENH91 remains to be elucidated.

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