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# Plasmid Dependence of *Pseudomonas* sp. Strain NK87 Enzymes That Degrade 6-Aminohexanoate-Cyclic Dimer

KAZUO KANAGAWA, SEIJI NEGORO,* NOBUO TAKADA, AND HIROSUKE OKADA

*Department of Fermentation Technology, Osaka University, Yamada-oka, Suita-shi, Osaka 565, Japan*

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**A bacterial strain, *Pseudomonas* sp. strain NK87, that can use 6-aminohexanoate-cyclic dimer as the sole source of carbon and nitrogen was newly isolated from wastewater of a factory which produces nylon-6. Two responsible enzymes, 6-aminohexanoate-cyclic-dimer hydrolase (P-EI) and 6-aminohexanoate-dimer hydrolase (P-EII), were found in the NK87 strain, as is the case with *Flavobacterium* sp. strain KI72, another 6-aminohexanoate-cyclic-dimer-metabolizing bacterium (H. Okada, S. Negoro, H. Kimura, and S. Nakamura, Nature [London] 306:203–206, 1983). The P-EI enzyme is immunologically identical to the 6-aminohexanoate-cyclic-dimer hydrolase of KI72 (F-EI). However, antiserum against the 6-aminohexanoate-dimer hydrolase purified from KI72 (F-EII) did not react with cell extracts of NK87, indicating that the F-EII and P-EII enzymes are immunologically different. Restriction endonuclease analyses show that the NK87 strain harbors at least six plasmids ranging in size from 20 to 80 kilobase pairs (kbp). The P-EI and P-EII genes were cloned in *Escherichia coli*. Both the P-EI and F-EI probes strongly hybridized with a 23-kbp plasmid in Southern hybridization analyses. The P-EII probe hybridized specifically with an 80-kbp plasmid, but the F-EII probe hybridized with none of the plasmids harbored in NK87. These results indicate that the P-EI gene and P-EII gene are encoded on the 23-kbp and 80-kbp plasmids, respectively.**

The recent development of the chemical industry has brought about the distribution of a wide variety of synthetic compounds. Enzymes responsible for the degradation of synthetic compounds provide us with a suitable system for studying how microorganisms acquire such specific abilities.

We have reported that *Flavobacterium* sp. strain KI72 metabolizes a by-product of the nylon-6 industry, 6-aminohexanoate-cyclic dimer (7), through two enzymes, 6 aminohexanoate-cyclic-dimer hydrolase (F-EI) (8) and 6-aminohexanoate-dimer hydrolase (F-EII) (9). These enzymes are encoded on plasmid pOAD2, one of the three plasmids harbored in KI72 (14). Both of the genes have been cloned in *Escherichia coli* (15), and their amino acid sequences were determined from their nucleotide sequences. F-EII enzyme is composed of 392 amino acid residues (16) and is active on 6-aminohexanoate oligomers (degree of polymerization, 2 to 20) and analogous substrates such as *N*-6-aminohexanoyl-8-aminooctanoic acid (9, 12). F-EI enzyme is composed of 493 amino acids (21) and is specifically active toward 6-aminohexanoate-cyclic dimer (8). To investigate how these genes are distributed in nature, we isolated a new bacterium that metabolizes the cyclic dimer. In this article, we report the plasmid dependence of the enzymes in a new isolate, *Pseudomonas* sp. strain NK87, and cloning of the genes.

## MATERIALS AND METHODS

**Microorganisms, plasmids, media, and culture conditions.** Bacterial strains that can grow with the 6-aminohexanoate-cyclic dimer as the sole carbon and nitrogen source were isolated from the wastewater of a nylon factory (Unitika, Uji, Japan) by the following procedure. The wastewater, suitably diluted with sterilized water, was spread directly on a cyclic-dimer minimum (CD) plate containing 2 g of 6-aminohexanoate-cyclic dimer, 3 g of $KH_2PO_4$, 1 g of $K_2HPO_4$, 2 g of NaCl, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.8 mg of $FeCl_3$, and 12 g of agar (pH 6.3) per liter. The plates were incubated at 30°C for 2 to 3 days. Colonies that appeared were further purified on CD plates. One of the isolates was named strain NK87. Cyclic dimer-yeast extract (CDY) medium (14) was used for cultivation of NK87. For cloning experiments, plasmids pUC12 (22) and pUC19 (24) were used as vectors, and *Escherichia coli* strains C600 $r_k^-$ $m_k^-$ (*thr-1 leuB6 thi-1 supE44 lacY1 tonA21 hsdM hsdR*) (1) and JM103 [Δ(*lac-pro*) *thi strA supE endA sbcB15 hsdR4* (F′ *traD36 proAB lacI*$^q$ *lacZΔM15*)] (11) were used as recipients for transformation.

**Preparation of crude enzyme solution of NK87.** NK87 cells grown in 100 ml of CDY medium were washed with 20 mM potassium phosphate buffer (pH 7.3) containing 10% glycerol (buffer I) and suspended in 5 ml of buffer I. The cell suspension was sonicated at 20 kHz for 8 min (Kaijo-Denki ultrasonicator, model 4280) and centrifuged at 10,000 × *g* for 5 min. The supernatant obtained was used as a crude enzyme solution.

**Assay.** The cell extracts obtained were incubated with 10 mM 6-aminohexanoate-cyclic dimer (for P-EI assay) or 10 mM 6-aminohexanoate-dimer (for P-EII assay) at 30°C for 45 min in buffer I, and the reaction products were detected by paper chromatography as described previously (8, 14). Protein content was measured from the $A_{280}$, assuming that 1 absorbance unit corresponds to 0.5 mg/ml.

**Immunological test.** Double immunodiffusion tests (17) were done on an Ouchterlony agar plate with antisera against F-EI (14) and F-EII (16) purified from *Flavobacterium* sp. strain KI72.

**Plasmid isolation.** Cells were grown in 1 liter of CDY medium to $10^9$ cells per ml, washed with 50 mM Tris hydrochloride–20 mM EDTA, and suspended in 200 ml of the same buffer. A 20-ml portion of Pronase solution (5 mg/ml) and 12 ml of 20% sodium dodecyl sulfate were added to the cell suspension, and the mixture was incubated at 37°C for 1 h. The pH was adjusted to 12.4 with 4 M NaOH, and the mixture was mixed with a stirrer for 5 min. The lysate was brought back to pH 8.5 by addition of 2 M Tris

* Corresponding author.

hydrochloride buffer (pH 7.0), and then NaCl was added to the lysate to a final concentration of 0.5 M. To remove proteins, the lysate was extracted twice with phenol and once with chloroform-isoamyl alcohol (24:1) as described before (10). The plasmid DNA solution (30 ml) was mixed with an equal volume of ethanol, and the DNA precipitated by centrifugation (9,000 × *g*, 10 min) was further purified by CsCl-ethidium bromide density gradient centrifugation as described before (14). Plasmids of *E. coli* were prepared by the alkaline extraction method of Birnboim and Doly (2), followed by CsCl-ethidium bromide density gradient centrifugation.

**Electrophoresis.** Agarose gel electrophoresis was done by the method of Maniatis et al. (10); 0.6 and 1% gels were used for fractionation of intact plasmids and restriction fragments, respectively. Bacteriophage λ DNA digested with *Hin*dIII was used as a size marker for restriction fragments (10). DNA fragments were recovered from agarose gels by the method of Vogelstein and Gillespie (23) with a Geneclean kit (Bio101, Inc.).

**Hybridization technique.** Southern hybridization (20) and colony hybridization (6) were done by the procedure described in an Amersham Co. Ltd. manual (Membrane Transfer and Detection Method). Plasmids pNDH501 (13) containing the F-EI gene (F-EIa probe) and pNL212Δ10 (15) containing the F-EII gene (F-EII probe) were labeled with [α-$^{32}$P]dCTP (400 Ci/mmol; ICN Chemical Co.) to $10^7$ cpm/μg by nick translation (19). The following fragments were also labeled in vitro with [α-$^{32}$P]dCTP to a specific activity of $1 \times 10^7$ to $3 \times 10^7$ cpm/μg by a multipriming labeling kit from Amersham Co.: a 2.0-kilobase-pair (kbp) *Hin*dIII-*Sal*I fragment containing the F-EI gene (F-EIb probe), a 0.34-kbp *Eco*RV fragment of pNDH501 included in the F-EI gene (F-EIc probe), and a 0.94-kbp *Eco*RI-*Eco*RV fragment of pNK2 (P-EII probe). Hybridizations were done at 60°C.

**Restriction enzyme digestion, ligation, and transformation.** Restriction endonuclease digestions and ligation were done by conventional methods (10). For cloning of the P-EI and P-EII genes, restriction fragments were ligated with pUC12. *E. coli* strains C600 $r_k^-$ $m_k^-$ and JM103 were transformed with the ligated DNA by the $CaCl_2$ method (3).

**Detection of P-EI and P-EII production in transformants.** Transformant cells were grown in LB medium containing ampicillin (50 μg/ml) for 16 h. Bacterial cells were harvested and resuspended in 100 μl of buffer I. The cell suspensions were frozen at −80°C and thawed at 30°C. After the freezing-thawing was repeated once, the cells were lysed by adding 10 μl of 10% Triton X-100. The supernatant obtained by centrifugation (10,000 × *g* for 5 min) was incubated at 30°C for 16 h with 10 mM 6-aminohexanoate-cyclic dimer (for P-EI) or 10 mM 6-aminohexanoate-dimer (for P-EII), and reaction products were detected by paper chromatography (8, 14).

**Enzymes and chemicals.** Restriction endonucleases (*Bam*HI, *Eco*RI, *Eco*RV, *Hin*cII, *Hin*dIII, *Mlu*I, *Pst*I, *Sal*I, and *Sma*I) and T4 DNA ligase were obtained from Toyobo Co. Ltd. 6-Aminohexanoate-dimer was chemically synthesized in our laboratory (7). 6-Aminohexanoate-cyclic dimer was a generous gift from Toyobo Co. Ltd. and was further purified by recrystallization from hot water (7). Other chemicals were purchased from Nakarai Chemical Co. Ltd.

## RESULTS

**Isolation of nylon oligomer-metabolizing bacterium NK87.** A strain, NK87, that can grow on 6-aminohexanoate-cyclic dimer as the sole source of carbon and nitrogen was isolated from wastewater of a factory (Unitika Co. Ltd) located at Uji, Kyoto, which produces nylon-6. This bacterium was rod shaped (size, 0.7 to 1.4 μm) and gram-negative and had a monopolar flagellum. The guanine-plus-cytosine content of the DNA of this bacterium was 59%. From taxonomic studies (18), we concluded that this bacterium was a *Pseudomonas* strain.

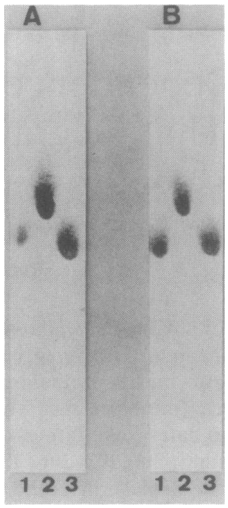


FIG. 1. Paper chromatography for detection of degradation products from 6-aminohexanoate-cyclic dimer and the linear dimer in crude enzyme extracts of *Pseudomonas* sp. strain NK87. Cell extracts of NK87 (15 mg/ml) were incubated with 10 mM 6-aminohexanoate-cyclic dimer (A) or 10 mM linear dimer (B) for 45 min. Reaction products were analyzed by paper chromatography. Slots: 1, reaction product; 2, authentic 6-aminohexanoate-dimer; 3, authentic 6-aminohexanoate.

**Enzymes responsible for degradation of nylon oligomer.** Cell extracts of strain NK87 produced a detectable amount of 6-aminohexanoate from 6-aminohexanoate-cyclic dimer and 6-aminohexanoate-dimer within 45 min of reaction (Fig. 1). This indicates that the NK87 cells have cyclic-dimer-hydrolyzing activity and linear-dimer-hydrolyzing activity, as is the case with *Flavobacterium* sp. strain KI72. The enzymes of NK87 were further examined by a double immunodiffusion test. Anti-F-EI serum formed clear precipitin lines with the cell extracts of NK87 (Fig. 2), which fused

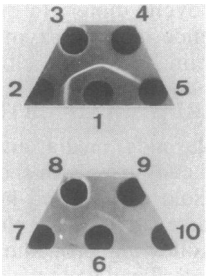


FIG. 2. Double immunodiffusion test between cell extracts of *Pseudomonas* sp. strain NK87 and antiserum; 35 μl each of enzyme and antiserum were put into each well. Well 1, Rabbit antiserum against the F-EI enzyme; wells 2 and 7, cell extracts of NK87 (80 μg); wells 3 and 8, cell extracts of NK87 (400 μg); wells 4 and 10, purified F-EI enzyme (2 μg); wells 5 and 9, purified F-EII enzyme (0.6 μg); well 6, rabbit antiserum against the F-EII enzyme.

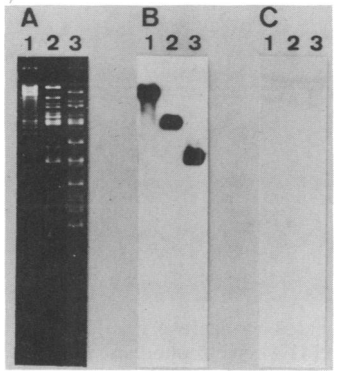


FIG. 3. Southern blot hybridization of restriction fragments of plasmids from *Pseudomonas* sp. strain NK87. Plasmids were purified from NK87 by CsCl-ethidium bromide density gradient centrifugation and digested with *Bam*HI (slots 1), *Eco*RI (slots 2), or *Hin*dIII (slots 3). The fragments obtained were fractionated by agarose gel (1%) electrophoresis (A). Southern blots of the gel were hybridized at 60°C with $^{32}$P-labeled F-EIa probe (nick-translated pNDH501) (B) or $^{32}$P-labeled F-EII probe (nick-translated pNL212Δ10) (C). Details are in Materials and Methods.

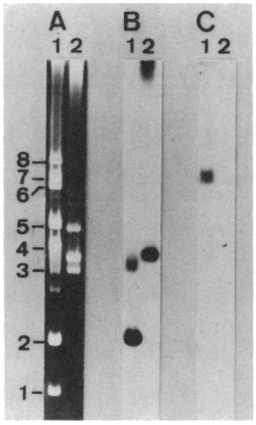


FIG. 4. Identification of plasmid encoding the P-EI and P-EII genes. Plasmids of *Pseudomonas* sp. strain NK87 (slots 1) and *Flavobacterium* sp. strain KI72 (slots 2) were purified by CsCl-ethidium bromide density gradient centrifugation and fractionated by agarose gel (0.6%) electrophoresis (A). Three plasmids harbored in KI72 are pOAD1 (39.7 kbp), pOAD2 (43.6 kbp), and pOAD3 (56.4 kbp). DNA bands (numbered 1 to 8) were recovered from the gel and used for experiments shown in Fig. 5. Southern blots of the gels were hybridized at 60°C with $^{32}$P-labeled F-EIc probe (0.34-kbp *Eco*RV fragment of pNDH501) (B) or with $^{32}$P-labeled P-EII probe (0.94-kbp *Eco*RI-*Eco*RV fragment of pNK2) (C).

at the corner with the precipitin line formed between the serum and purified F-EI enzyme. Thus, F-EI enzyme and the 6-aminohexanoate-cyclic-dimer hydrolase of *Pseudomonas* sp. strain NK87 (P-EI) are immunologically identical. On the contrary, no precipitin lines were observed between the cell extracts of NK87 and anti-F-EII serum, suggesting that the 6-aminohexanoate-dimer hydrolase of NK87 (P-EII) is immunologically different from the F-EII enzyme. Though the possibility remains that the content of P-EII in the cell extracts is too low to form a precipitin line against the anti-F-EII serum, Southern hybridization and cloning experiments (described below) indicate that these two enzymes show great differences in structure.

**Plasmids harbored in NK87.** CsCl-ethidium bromide density gradient centrifugation of NK87 DNA showed the presence of covalently closed circular DNA in the NK87 cells (data not shown). Restriction endonuclease digestion of covalently closed circular DNAs produced discrete bands during agarose gel electrophoresis (Fig. 3A). After DNA fragments in the gel were transferred to a nylon membrane by the Southern blotting technique, DNA fragments that contained nucleotide sequences homologous to F-EI and F-EII genes were examined by $^{32}$P-labeled probe DNAs. The F-EIa probe (nick-translated pNDH501; see Fig. 6) strongly hybridized with a 23-kbp *Bam*HI fragment, a 9.2-kbp *Eco*RI fragment, and a 5.2-kbp *Hin*dIII fragment (Fig. 3B). However, no hybridized fragments were detected in these plasmid fractions with the F-EII probe (nick-translated pNL212Δ10) (Fig. 3C). In contrast to the plasmids of *Flavobacterium* sp. strain KI72, which harbors three plasmids (pOAD1, pOAD2, and pOAD3) (Fig. 4A, slot 2) (14), more than eight bands were observed for the plasmid preparation of NK87 (Fig. 4A, slot 1). To identify these plasmids, we divided the agarose gels into eight fractions (Fig. 4A) and extracted the DNA, followed by digestion with *Pst*I (Fig. 5A) or *Hin*dIII (Fig. 5B). Different restriction patterns were observed except for fractions 2 and 3. Since the DNAs from fractions 2 and 3 yielded the same size fragments (21- and 2-kbp fragments) on *Pst*I digestion, the plasmid included in fraction 3 seems to be a multimer or open circular form of the plasmid included in fraction 2. Since contamination by smaller plasmids in fraction 8 brought ambiguity in specification of restriction fragments, we could not conclude that the plasmid included in fraction 8 is different from faster-migrating plasmids. The size of each plasmid was estimated by summing up the unique *Pst*I fragments (or *Hin*dIII fragments) for each plasmid. From these data, we tentatively named the plasmids as follows: pNAD1 (20 kbp), mainly included in fraction 1; pNAD2 (23 kbp) in fractions 2 and 3; pNAD3 (51 kbp) in fraction 4; pNAD4 (57 kbp) in fraction 5; pNAD5 (76 kbp) in fraction 6; and pNAD6 (80 kbp) in fraction 7.

**Cloning of the P-EI and P-EII genes.** Cloning of the P-EI and P-EII genes is essential for fine investigation of these genes. Since Southern hybridization experiments had revealed that the 5.2-kbp *Hin*dIII fragments hybridized to the F-EIa probe (Fig. 3B, slot 3), the plasmids purified by CsCl-ethidium bromide density gradient centrifugation were digested with *Hin*dIII. The DNA fragments obtained were fractionated by 1% agarose gel electrophoresis; 5.2-kbp fragments were recovered and ligated with pUC12 that had been cleaved by *Hin*dIII. *E. coli* JM103 was transformed with the ligated DNA, and the transformants were selected as white colonies on LB medium containing ampicillin (50 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (40 μg/ml), and isopropyl-β-D-thiogalactoside (IPTG) (0.1 mM) (11). *E. coli* clones harboring the P-EI gene were selected by colony hybridization with the F-EIb probe. Among the 50 clones obtained, plasmid DNAs were prepared from 4 clones selected independently. These plasmids showed the same restriction pattern for *Eco*RI, *Hin*dIII, and *Pst*I digestions, and we selected one (pNK1) for further study. Single and double digestions of pNK1 by various restriction endonucleases enabled us to make a restriction map of the plasmid (Fig. 6). A deletion plasmid was constructed from pNK1 to locate the P-EI gene. Plasmid pNK11 was constructed by connecting the 2.2-kbp *Sal*I fragment to pUC19 that had been cleaved by *Sal*I. pNK11R, in which the 2.2-kbp fragment was connected to the vector region in reverse orientation, showed no P-EI expression. These results suggest that

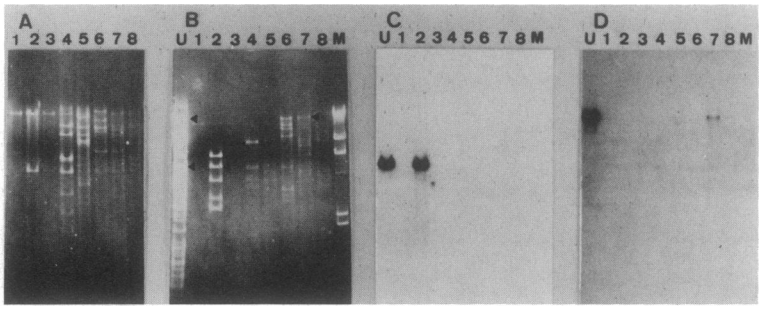


FIG. 5. Southern blot hybridization of fractionated plasmids. Plasmids were fractionated by agarose gel (0.6%) electrophoresis and digested with *Pst*I (A) or *Hin*dIII (B to D). The fragments were separated by agarose gel (1%) electrophoresis, followed by staining with ethidium bromide (A and B). Plasmids encoding the P-EI or P-EII gene were identified by Southern blot hybridizations with $^{32}$P-labeled F-EIc probe (C) and $^{32}$P-labeled P-EII probe (D), respectively. Slots 1 to 8, Plasmid samples recovered from fractions 1 to 8 in Fig. 4, respectively. Slot U, Unfractionated plasmids of NK87; slot M, λ phage DNA digested with *Hin*dIII. Fragments detected by the F-EIc and P-EII probes are marked by triangles (B).

the P-EI gene is expressed under the control of the *lac* promoter included in pUC19 (Fig. 6).

Clones having the P-EII gene cannot be selected by colony hybridization with a F-EII probe. Thus, we attempted to isolate the gene by observing which clones contained 6-aminohexanoate-dimer hydrolase activity (see Materials and Methods). DNA fragments prepared from unfractionated plasmids (mixture of pNAD1-6) by partial digestion with *Pst*I were ligated to pUC12 which had been digested with *Pst*I, and ampicillin-resistant white transformants were selected in a way similar to that used for isolation of P-EI clones. Cell extracts from 3 of the 50 clones produced 6-aminohexanoate from its dimer after 16 h, indicating the presence of cloned P-EII genes. *Pst*I digestion of the plasmids recovered from one of the three (named pNK2) contained the 2.5-kbp fragment and 2.6-kbp vector fragment. The hybrid plasmids of another two clones contained the same 2.5-kbp fragment and some additional fragments: 6.5-kbp fragment in one clone, and 6.5- and 0.8-kbp fragments in the remaining one. To locate the P-EII gene on pNK2, deletion plasmids were constructed from pNK2 (Fig. 7). The linear-dimer-hydrolyzing activity was detected in *E. coli* harboring pNK2S2, in which a 0.55-kbp *Eco*RI fragment was lost, but not in pNK2S3, in which a 0.8-kbp *Eco*RV-*Sma*I fragment was deleted. Thus, one end of the structural gene is between the *Eco*RI and *Eco*RV sites. Since the enzyme activity could not be detected in *E. coli* having pNK2S5 that lacks the opposite end of the inserted fragment (0.82-kbp *Pst*I-*Eco*RI fragment), the 0.94-kbp *Eco*RI-*Eco*RV fragment used as the P-EII probe (described below) should be included in the P-EII gene.

**Identification of plasmids encoding the P-EI and P-EII genes.** To identify the plasmid which encodes these genes, plasmid DNAs fractionated by agarose gel electrophoresis were hybridized with $^{32}$P-labeled probes specific for the P-EI and P-EII genes. Southern hybridization experiments showed that a 0.34-kbp *Eco*RV fragment of plasmid pNDH501 completely included in the F-EI gene (F-EIc probe, amino acid positions 82 to 194) (21) strongly hybridized with the two bands that had been identified as plasmid pNAD2 (Fig. 4B, slot 1). Southern hybridization against the fractionated plasmid digested with *Hin*dIII demonstrated that the F-EI probe hybridized specifically with a 5.2-kbp *Hin*dIII fragment which was derived from pNAD2 (Fig. 5C, slots U and 2). Similar hybridization was done with a 0.94-kbp *Eco*RI-*Eco*RV fragment of pNK2 (P-EII probe). This probe specifically hybridized with a band (fraction 7) identified as plasmid pNAD6 (Fig. 4C, slot 1) and a 10-kbp

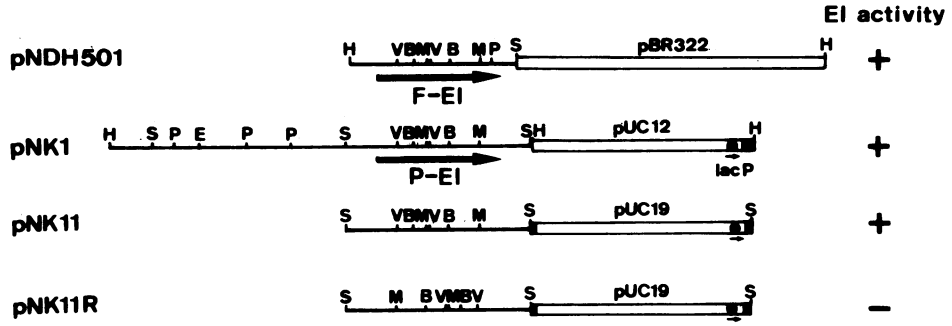


FIG. 6. Restriction map of plasmids containing the P-EI gene. Plasmid pNK1 was composed of vector pUC12 and the 5.2-kbp *Hin*dIII fragment of pNAD2. The 2.2-kbp *Sal*I fragment of pNK1 was cloned into the unique *Sal*I site of pUC19; pNK11 and pNK11R, in which the 2.2-kbp fragment was connected to the vector in reverse orientation, were obtained. Plasmid pNDH501 is composed of the 2.0-kbp *Hin*dIII-*Sal*I fragment containing the F-EI gene and the 3.7-kbp *Hin*dIII-*Sal*I region of pBR322. B, E, H, M, P, V, and S indicate restriction sites for *Bam*HI, *Eco*RI, *Hin*dIII, *Mlu*I, *Pst*I, *Eco*RV, and *Sal*I, respectively. Solid boxes indicate multilinkers containing restriction sites for *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, and *Sal*I (11, 24). Restriction sites in vector and multilinker regions are omitted. The length and direction of an arrow indicate gene size expected from the molecular weight of the subunit of the EI enzyme and direction of the expression of the gene, respectively. Cell extracts of *E. coli* C600 $r_k^-$ $m_k^-$(pNDH501), JM103(pNK1), JM103(pNK11), and JM103(pNK11R) were incubated with 10 mM 6-aminohexanoate-cyclic dimer at 30°C for 16 h, and production of 6-aminohexanoate-dimer was examined by paper chromatography.

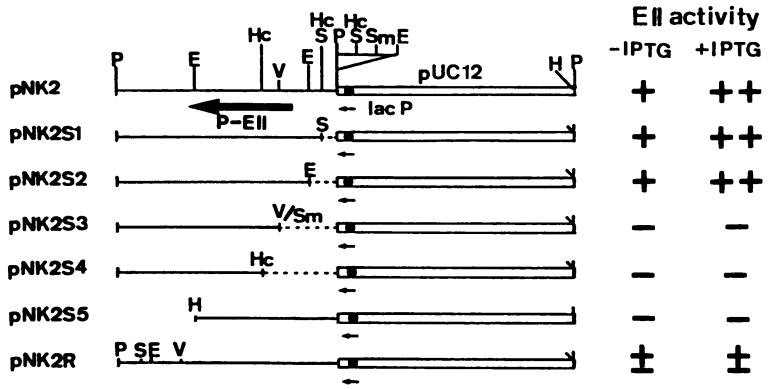


FIG. 7. Restriction map of plasmids containing the P-EII gene. Plasmid pNK2 was composed of vector pUC12 and the 2.5-kbp *Pst*I fragment of pNAD6. To construct miniplasmids, pNK2 was digested with restriction endonucleases, followed by ligation and transformation of *E. coli* JM103. pNK2S1 was obtained by complete *Sal*I digestion, pNK2S2 by partial *Eco*RI digestion, pNK2S3 by complete *Eco*RV-*Sma*I digestion, and pNK2S4 by complete *Hin*cII digestion. For construction of pNK2S5, pNK2 was digested completely with *Hin*dIII and then partially with *Eco*RI. After the terminal cohesive ends were filled in with *E. coli* DNA polymerase I (Klenow fragment), the fragment having the flush ends was purified by agarose gel (1%) electrophoresis. The fragment obtained was ligated with phosphorylated *Hin*dIII linker (CAAGCTTG), followed by *Hin*dIII digestion and ligation. After pNK2 was digested with *Pst*I, followed by ligation and transformation, plasmid pNK2R, in which the 2.5-kbp *Pst*I fragment was connected to the vector region in reverse orientation, was obtained. E, H, P, V, and S are defined in the legend to Fig. 6. Hc and Sm indicate restriction sites for *Hin*cII and *Sma*I, respectively. For qualitative detection of the EII activity, *E. coli* JM103 harboring each plasmid was cultivated on LB medium (−IPTG) or LB medium containing 0.1 mM IPTG (+IPTG). Cell extracts obtained by freeze-thawing and Triton X-100 treatments were incubated with 10 mM 6-aminohexanoate-dimer at 30°C for 2 to 16 h, and production of 6-aminohexanoate was examined by paper chromatography (details are described in the text). Symbols: ++, 70 to 90% hydrolysis in 2 h of reaction; +, 70 to 90% hydrolysis in 6 h of reaction, ±, 5 to 10% hydrolysis in 16 h of reaction; −, no hydrolysis even after 16 h of reaction. Size and direction of expression of the gene are shown as arrows.

*Hin*dIII fragment derived from pNAD6 (Fig. 5D, slots U and 7). From these results, we concluded that the P-EI and P-EII genes are present on plasmids pNAD2 and pNAD6, respectively.

## DISCUSSION

The genes for the 6-aminohexanoate-cyclic-dimer hydrolase from *Flavobacterium* and *Pseudomonas* spp. (F-EI and P-EI) seem to be highly homologous: (i) the F-EI and P-EI enzymes are immunologically identical (Fig. 2); (ii) the F-EI probe hybridized to the P-EI gene at 60°C with almost the same intensity as it did to the F-EI gene (Fig. 4); and (iii) six restriction sites (two *Bam*HI sites, two *Eco*RV sites, and two *Mlu*I sites) are conserved in both genes (Fig. 6). However, it also should be noted that these F-EI and P-EI enzymes are not identical, since the *Pst*I site on the F-EI gene is absent from the P-EI gene (Fig. 6). Recently, we found that F-EI and P-EI enzymes have 98.6% homology in their amino acid sequences (21).

In contrast to the high homology of the F-EI and P-EI genes, F-EII and P-EII show greater differences in their structure: (i) the F-EII probe did not hybridize with the P-EII gene (Fig. 3C); (ii) similarly, the P-EII probe showed no hybridization toward the F-EII gene (Fig. 4C); and (iii) antigenic protein reactive with anti-F-EII serum could not be detected in the cell extract of NK87. DNA sequencing of the cloned P-EII gene revealed that the F-EII and P-EII enzymes have 35% homology in their amino acid sequences (unpublished results). Thus, F-EII and P-EII enzymes appear to be evolutionarily related. However, these two probably branched far earlier from their ancestral enzymes than the F-EI and P-EI enzymes did in their evolution.

Genes responsible for the degradation of organic compounds such as toluene, naphthalene, salicylate, camphor, and *n*-alkane are localized as a cluster on the TOL, NAH, SAL, CAM, and OCT plasmids, respectively (5). In contrast to the close localization of these genes, the F-EI and F-EII genes of *Flavobacterium* sp. strain KI72 are 15.7 kbp apart on plasmid pOAD2. Interestingly, in *Pseudomonas* sp. strain NK87, the genes essential for the degradation of 6-aminohexanoate-cyclic dimer are present on different plasmids. This result indicates that microorganisms are able to construct a sequential metabolic pathway of a xenobiotic compound by acquiring several plasmids encoding either enzyme in the metabolic pathway. Once all of the responsible genes are present into one microorganism, these genes have a chance to be assembled into a single discrete unit through genetic rearrangements such as recombination and transposition. This process might be essential for evolving a new degradative plasmid, as proposed previously (4).

Our attempt to eliminate the P-EI and P-EII activities by curing treatments has been unsuccessful. Even after NK87 cells were cultivated in the presence of various agents, such as mitomycin (10 μg/ml), sodium dodecyl sulfate (20 μg/ml), ethidium bromide (160 μg/ml), acridine orange (200 μg/ml), rifampin (10 μg/ml), and novobiocin (10 μg/ml), or cultivated at 42°C, the cyclic-dimer-metabolic activity was retained in about 3,000 clones which were isolated independently. The high stability may be a great advantage for NK87 cells growing in the wastewater of a nylon factory, from which this microorganism was isolated.

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