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Plasmid-Determined Enzymatic Degradation of Nylon Oligomers

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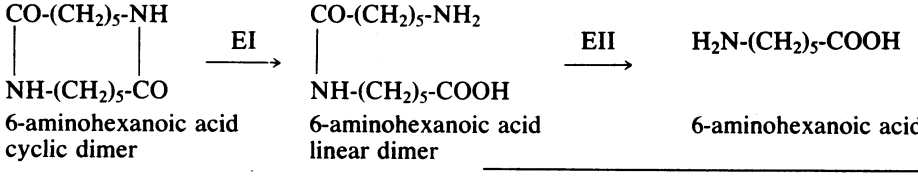
The nylon oligomer (6-aminohexanoic acid cyclic dimer) degradation genes on plasmid pOAD2 of *Flavobacterium* sp. KI72 were cloned into *Escherichia coli* vector pBR322. The locus of one of the genes, the structural gene of 6-aminohexanoic acid linear oligomer hydrolase, was determined by constructing various deletion plasmids and inserting the *lac*UV5 promoter fragment of *E. coli* into the deletion plasmid. Two kinds of repeated sequences (RS-I and RS-II) were detected on pOAD2 by DNA-DNA hybridization experiments. These repeated sequences appeared five times (RS-I) or twice (RS-II) on pOAD2. One of the RS-II regions and the structural gene of the hydrolase overlapped.

Since the middle of this century, the rapid progress of the chemical industry has led to the distribution of a wide variety of synthetic compounds as industrial products and wastes. One example is 6-aminohexanoic acid cyclic dimer, a by-product of nylon-6 manufacture. Our isolation of a bacterium, *Flavobacterium* sp. KI72, which can grow on the cyclic dimer as a sole source of carbon and nitrogen (8) raised the question of how the microorganisms evolved the capacity to degrade a synthetic compound that has appeared only since industrialization. Two new enzymes, 6-aminohexanoic acid cyclic dimer hydrolase (EI) (9) and 6-aminohexanoic acid linear oligomer hydrolase (EII) (10), were found to be responsible for the metabolism of the dimer, and the sequence of reaction was determined to be as follows:



6-aminohexanoic acid cyclic dimer — 6-aminohexanoic acid linear dimer — 6-aminohexanoic acid

The cyclic and linear oligomer hydrolases, purified to homogeneity, were active only toward the cyclic dimer and linear oligomer, respectively, and inactive toward the more than 100 natural linear and cyclic amide bonds tested. Enzymes active toward synthetic compounds but not natural compounds had no reason to exist before the creation of the synthetic compounds. The enzymes are encoded on a plasmid, pOAD2, harbored in strain KI72. Here we describe the determination of the fine locus of one of the nylon oligomer degradation genes, EII, and the detection on the same plasmid of a DNA region homologous to part of the EII structural gene.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Flavobacterium* sp. KI72 {harboring three plasmids, pOAD1 (39.7 kilobase pairs [kbp]), pOAD2 (43.6 kbp), and pOAD3 (56.4 kbp)}, was isolated from the wastewater of a nylon-6 factory. KI723T1(pOAD11, pOAD2, pOAD3) is a strain derived from KI723(pOAD11, pOAD3) (a mitomycin C-treated, pOAD1-partly deleted, and pOAD2-cured strain of KI72) by transformation with a plasmid-DNA mixture of the wild-type strain (12). *Escherichia coli* C600 *thr-1 leu-6 thi-1 supE44 lacY1 tonA21 hsdM hsdR* (1) and strain 294 (*endoI thi hsdR*) (2) were used as recipients for cloning experiments. Plasmid pBR322, which carried ampicillin and tetracycline resistance markers, was used as a cloning vector (3).

Media and culture conditions. The medium used for the growth of *Flavobacterium* sp. was a cyclic dimer-yeast extract broth containing 8 g of 6-aminohexanoic acid cyclic dimer, 1 g of K_2HPO_4, 3 g of KH_2PO_4, 5 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 3 mg of $FeCl_3$, and 0.5 g of yeast extract in 1 liter (pH 6.4). For the growth of *E. coli*, Luria broth containing 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 1 g of glucose in 1 liter (pH 7.2) was used.

Enzymes. Restriction endonucleases *Eco*RI, *Hin*dIII, *Bam*HI, *Sal*I, *Pst*I, *Bgl*II, *Mul*I, *Pvu*II, *Sma*I, and *Xho*I and T4 DNA ligase were purchased from Takara Brewery Co. Ltd., Kyoto, Japan. Bacterial alkaline

phosphatase type III-R was obtained from Sigma Chemical Co., St. Louis, Mo.

Plasmid isolation. Plasmid DNAs of *Flavobacterium* sp. were prepared by CsCl-ethidium bromide density gradient centrifugation as described previously (12). To isolate pOAD2 from other plasmids harbored in the *Flavobacterium* sp., the plasmid mixture obtained by density gradient centrifugation was subjected to electrophoresis through agarose gel. After staining the gel with ethidium bromide (1 μg/ml), the corresponding band was cut out and extracted as described in a previous paper (12).

Restriction endonuclease digestion. Restriction of plasmid DNAs was carried out at 37°C for 1 to 3 h in the following buffers: 100 mM Tris-hydrochloride (pH 7.5)–50 mM NaCl–10 mM $MgCl_2$ for *Eco*RI; 6 mM Tris-hydrochloride (pH 7.5)–50 mM NaCl–10 mM $MgCl_2$–100 μg of bovine serum albumin per ml for *Hin*dIII; 100 mM Tris-hydrochloride (pH 7.5)–10 mM $MgCl_2$ for *Bam*HI; 8 mM Tris-hydrochloride (pH 7.5)–150 mM NaCl–8 mM $MgCl_2$–100 μg of bovine serum albumin per ml for *Sal*I; 20 mM Tris-hydrochloride (pH 7.5)–10 mM $MgCl_2$–50 mM $(NH_4)_2SO_4$–100 μg of bovine serum albumin per ml for *Pst*I; 20 mM Tris-hydrochloride (pH 7.5)–7 mM $MgCl_2$–7 mM β-mercaptoethanol for *Bgl*II; 10 mM Tris-hydrochloride (pH 7.5)–100 mM NaCl–7 mM $MgCl_2$–7 mM β-mercaptoethanol for *Mlu*I; 6 mM Tris-hydrochloride (pH 7.5)–60 mM NaCl–6 mM $MgCl_2$–6 mM β-mercaptoethanol–100 μg of bovine serum albumin per ml for *Pvu*II; and 8 mM Tris-hydrochloride (pH 7.5)–150 mM NaCl–6 mM $MgCl_2$–6 mM β-mercaptoethanol for *Xho*I. *Sma*I digestion was carried out at 30°C for 1 to 3 h in 15 mM Tris-hydrochloride buffer (pH 8.0) containing 6 mM $MgCl_2$ and 15 mM KCl. Digestion was terminated by heating at 65°C for 10 min. In double-digestion experiments, the endonuclease reaction requiring a low salt concentration was carried out first, and then after addition of extra salts the second endonuclease digestion was started.

Construction of recombinant plasmids. pOAD2 DNA (1.5 μg) was partially digested with 1 U of *Hin*dIII at 37°C for 1 h in 50 μl of the reaction buffer described above. The reaction was terminated by heating at 65°C for 20 min. pBR322 DNA (30 μg) which had been linearized with *Hin*dIII was incubated with 3 U of bacterial alkaline phosphatase in 150 μl of 25 mM Tris-hydrochloride (pH 8.0) at 65°C for 30 min to remove the phosphate residues at the 5′ terminals (15). By this treatment the self-ligation frequency of vector DNA was greatly decreased.

Partially digested pOAD2 DNA (1.5 μg) and phosphatase-treated pBR322 DNA (0.5 μg) were ligated in 50 μl of a reaction mixture containing 77 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, 1 mM ATP, 10 mM dithiothreitol, and 0.2 U of T4 DNA ligase per ml at 4°C for 16 h.

Transformation. Transformation was carried out according to the procedure of Cohen et al. (5), using *E. coli* C600 or 294 as the recipient. Transformants selected for ampicillin resistance were subjected to replica plating on Luria broth plates, each containing 50 μg of ampicillin and 30 μg of tetracycline per ml. Colonies which were ampicillin resistant and tetracycline sensitive, which accounted for more than 80% of the total ampicillin-resistant colonies, were selected.

Detection of EI and EII productivity of the transformants. Transformant cells grown on Luria broth (4 ml) containing ampicillin (50 μg/ml) to a density of 2×10^9 cells per ml were harvested by centrifugation, washed with 20 mM potassium phosphate buffer (pH 7.4) containing 10% glycerol, and suspended in 0.2 ml of the same buffer. The cell suspension was supplemented with 1 mg of lysozyme per ml and kept at 0°C for 30 min. Cell lysis was completed by the addition of 1% Triton X-100, and the lysate was used as the crude enzyme solution. Crude enzyme solution (0.2 ml) was added to 0.2 ml of 20 mM 6-aminohexanoic acid cyclic dimer (for the detection of EI activity) or 20 mM 6-aminohexanoic acid linear dimer (for the detection of EII activity), and the mixtures were incubated at 30°C for 16 h. Portions of 5 μl each of the reaction mixtures were subjected to paper chromatography, and the reaction products were detected with ninhydrin reagent as described previously (8).

Electrophoreses. Vertical agarose slab gel electrophoreses of plasmid DNA and the restriction fragments were conducted as described previously (12). Fragments of λ phage DNA generated by *Hin*dIII digestion were used as molecular weight standards (7).

Preparation of DNA probes. Plasmid DNA (pNDH2912, pNDH10, or pNDH513) (1 μg) or the *Bam*HI fragment with 1.58 kbp from pNL21 (1 μg) was labeled by nick translation (13) to a specific activity of 5×10^6 to 2×10^7 cpm/μg with [α-^{32}P]dCTP (400 Ci/mmol; Amersham Corp., Arlington Heights, Ill.).

Blotting and hybridization procedures. DNA fragments obtained by restriction endonuclease digestion were transferred from the agarose gel to a nitrocellulose filter (type HAWP; Millipore Corp., Bedford, Mass.) and hybridized to a radioactive probe by the method of Southern (14). The transfer buffer was 20 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M trisodium citrate). After transfer the filters were rinsed with 2 × SSC, air dried, and then baked at 80°C for 4 h. Before hybridization the nitrocellulose filter was incubated at 65°C in 150 ml of 3 × SSC for 30 min, then 150 ml of 3 × SSC containing 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, and 0.2% Ficoll 400 (buffer I) for 3 h, and finally in 100 ml of buffer II (buffer I containing 50 μg of denatured calf thymus DNA per ml and 0.1% sodium dodecyl sulfate) for 1 h. After pretreatment the filter was dried and placed in a plastic bag with 4 ml of hybridization solution (buffer II containing 50 ng of ^{32}P-labeled DNA per ml [2×10^6 total cpm]). The plastic bag was sealed and immersed at 65°C for 20 h. After hybridization, the nitrocellulose filter was washed with buffer II six times (8 min each) at 65°C and then washed with 3 × SSC twice at room temperature. After the filter was airdried, it was exposed to Kodak X-Omat S X-ray film at −80°C for 16 to 72 h.

RESULTS

Cloning of nylon oligomer degradation genes in *E. coli*. The wild-type strain KI72 harbored three kinds of plasmids, pOAD1, pOAD2, and pOAD3, two of which, pOAD1 and -2, are inseparable by agarose gel electrophoresis because of their similar sizes (12). We therefore prepared pOAD2 plasmid DNA from KI723T1 (pOAD11, pOAD2, pOAD3). pOAD11 is a dele-

tion plasmid of pOAD1 and is separable from pOAD2 by agarose gel electrophoresis.

pOAD2 gave six *Hin*dIII fragments of 12.7 (A), 12.0 (B), 9.1 (C), 5.8 (D), 3.3 (E), and 0.83 (F) kbp (Fig. 1) (11). The pOAD2 fragments obtained by partial digestion with *Hin*dIII were ligated to a vector, pBR322, which had been cleaved by *Hin*dIII and treated with alkaline phosphatase. After transformation of *E. coli* with the hybrid plasmid, ampicillin-resistant, tetracycline-sensitive clones were selected. To analyze the EI and EII enzyme productivities of the selected transformants, cell extracts were incubated with the cyclic or linear dimer, and the reaction products were detected by paper chromatography. Cell extracts from six of the 36 clones tested produced the linear dimer from the cyclic dimer, indicating the presence of the cloned EI gene. Cell extracts of another five clones produced the monomer from the linear dimer, indicating the presence of the cloned EII gene. No clones producing both EI and EII enzymes were detected. Restriction endonuclease (*Hin*dIII, *Eco*RI, and *Hin*dIII+*Eco*RI) analyses of the plasmids harbored in EI-producing clones revealed that all of them were composed of the vector DNA and the *Hin*dIII-C fragment of pOAD2 (data not shown). The hybrid plasmid harbored in the five EII-producing transformants contained the *Hin*dIII-A fragment in two cases, the A+F fragments in another two, and the D+A+F fragments in the remaining one. In clones producing neither EI nor EII, hybrid plasmids contained the *Hin*dIII-B, -B+E, -D, or -E fragment. From these results we concluded that the structural genes for EI and EII are located, respectively, on the *Hin*dIII-C and -A fragments of pOAD2.

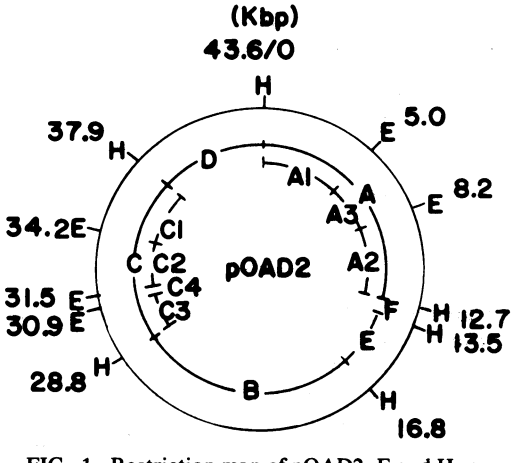


FIG. 1. Restriction map of pOAD2. E and H represent the *Eco*RI and *Hin*dIII sites, respectively (11).

EII gene expression under the control of the *lac*UV5 promoter. The hybrid plasmid carrying the pOAD2-A fragment containing the EII gene was named pNDH29. To locate the promoter site controlling the EII gene, pNDH29 digested with *Hin*dIII were rejoined with T4 DNA ligase, this treatment reversing the orientation of the foreign DNA in half of the resultant hybrid plasmids (Fig. 2). Both types of plasmids (pNDH29 and pNDH292) showed similar EII enzyme expression, indicating that the promoter site lies within the foreign DNA. Various deletion plasmids of pNDH29 were prepared as shown in Fig. 2. Deletion of the A3 fragment from pNDH29 did not affect EII enzyme production, whereas deletion of either the A1 or the A2 fragment suppressed it completely. To test the possibility that the structural gene is located on the A1 fragment and is under the control of a promoter located on the A2 fragment, we replaced the A2 fragment with *lac*UV5 promoter (6) by introducing the 207-bp *Eco*RI fragment derived from pKB252 (2), which encodes this promoter, at the *Eco*RI site of pNDH2912, which consists of pBR322 and the A1 fragment of pOAD2. After transformation of *E. coli* 294 cells (2) with this hybrid plasmid, ampicillin-resistant, β-galactosidase-constitutive clones were selected. Of 60 clones, 26 produced about 10 times more EII enzyme (0.002 U/mg of protein) than the same strain harboring pNDH29, and 34 produced none. Restriction endonuclease (*Pvu*II, *Pst*I, and *Pst*I+*Sma*I) analyses of the plasmids harbored in EII-positive transformants revealed that the promoter was joined with a certain orientation to the A1 fragment. An EII-negative transformant tested harbored a plasmid in which the *lac* promoter was connected in the opposite direction to the A1 fragment. In a parallel experiment, the introduction of the *lac* promoter into a plasmid (pNDH2921) consisting of the vector DNA and the A2 region failed to express the EII gene, irrespective of the promoter's orientation. These results suggest that the EII gene is under the control of a promoter located in the A2 region, although the possibility remains that a factor encoded in the A2 region positively regulates the EII gene.

To determine the precise locus of the EII gene, deletion plasmids were constructed from plasmid pNL2, which encodes the A1 fragment, the *lac*UV5 promoter, and pBR322 (see Fig. 2). The smallest EII gene-expressible miniplasmid derived from it was pNL212Δ10, which contained DNA from pOAD2 mapped between 2.7 (a *Bam*HI site) and 4.1 kbp (see Fig. 2). Since this size agrees closely with that expected from the molecular weight of the EII enzyme subunit (42,000) (10) and the EII activity was lost by deleting a 70-bp *Bam*HI fragment (mapped in the

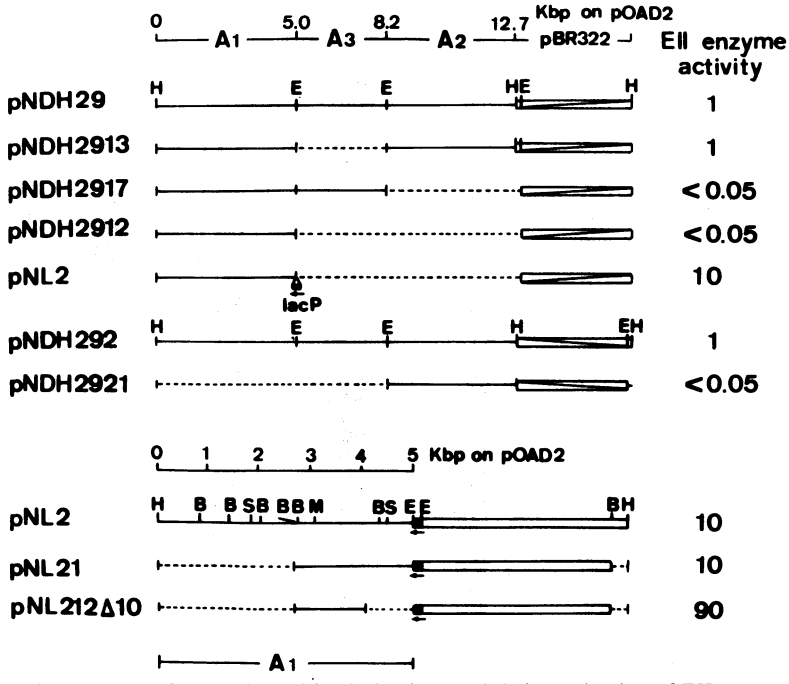


FIG. 2. Plasmid structure of pNDH29 and its derivatives and their production of EII enzyme. To construct miniplasmids of pNDH29, the plasmid was partially digested with *Eco*RI, followed by ligation and transformation. pNDH2913, -2917, and -2912 lacked the A3, A2, and both A2 and A3 regions, respectively, as shown by broken lines. pNL2 is a plasmid derived from pNDH2912 by introducing the 207-bp *lac*UV5 promoter at the *Eco*RI site in the proper orientation. The heavy bar indicates the promoter fragment. pNL21, a miniplasmid of pNL2, was constructed by partial *Bam*HI digestion of pNL2 followed by ligation and transformation. After digestion of the pNDH29 with *Hin*dIII followed by ligation and transformation, plasmid pNDH292, in which the *Hin*dIII-A fragment was connected to the vector DNA in the reverse orientation, was obtained. Plasmid pNDH2921, which was derived from pNDH292 by complete *Eco*RI digestion followed by ligation and transformation, lacked both A1 and A3 fragments. Plasmid pNL21 was further modified to eliminate the *Eco*RI site distal to the *lac* promoter (the right *Eco*RI site), and pNL212 was obtained. pNL212 was digested with *Sma*I at a site located at the middle of the structural gene and the promoter. Then it was digested with exonuclease III followed by nuclease S1. To make the upstream side of the DNA uniform, the resulting DNA was treated with *Eco*RI and T4 DNA polymerase. Both flush ends of the DNA molecules were ligated and transformed to *E. coli* 294, and plasmid pNL212Δ10, which lacks the 4.1- to 5.0-kbp region, was obtained. For qualitative detection of EII enzyme activity, extracts of cells (*E. coli* C600 or 294 harboring each plasmid) obtained by lysozyme-Triton X-100 treatment were added to 20 mM 6-aminohexanoic acid linear dimer solution and incubated at 30°C. Enzyme activities were determined by densitometric analyses of a spot of 6-aminohexanoic acid produced from the linear dimer after paper chromatography. Enzyme activities were expressed as the ratio toward the activity of *E. coli* C600 harboring pNDH29. B, E, H, M, and S represent *Bam*HI, *Eco*RI, *Hin*dIII, *Mlu*I, and *Sma*I sites, respectively.

2.7 to 2.8-kbp region) from pNL212Δ10, the locus of the EII structural gene was concluded to be from 2.7 to 4.1 kbp on pOAD2.

Location of the DNA regions homologous to the pOAD2-A1 fragment. The plasmids or chromosomal DNAs of *Flavobacterium* sp. KI72 and its derivative strains were digested with restriction endonucleases, and the resultant DNA fragments were fractionated by agarose gel electrophoreses and transferred to nitrocellulose filters by the Southern blotting technique (14). Figure 3 shows the Southern hybridization of pOAD2 fragments with pNDH2912 containing the EII structural gene (see Fig. 2) as the probe. Three *Hin*dIII fragments of pOAD2 (B [16.8- to 28.8-kbp region], C [28.8 to 37.9 kbp], and E [13.5 to 16.8 kbp]) were detected as hybridized fragments. Although some minor radioactive bands can be seen, we ignored those additional bands which did not correspond to visible stained bands as artifacts or incomplete digestion products. In control experiments with pBR322 as the probe, no hybridized fragments could be detected among pOAD2 fragments. To determine the hybridized region more precisely, pNDH6 (a hybrid plasmid consisting of pBR322 and the *Hin*dIII-B fragment of pOAD2), pNDH5 (pBR322 + fragment C), and pNDH10 (pBR322

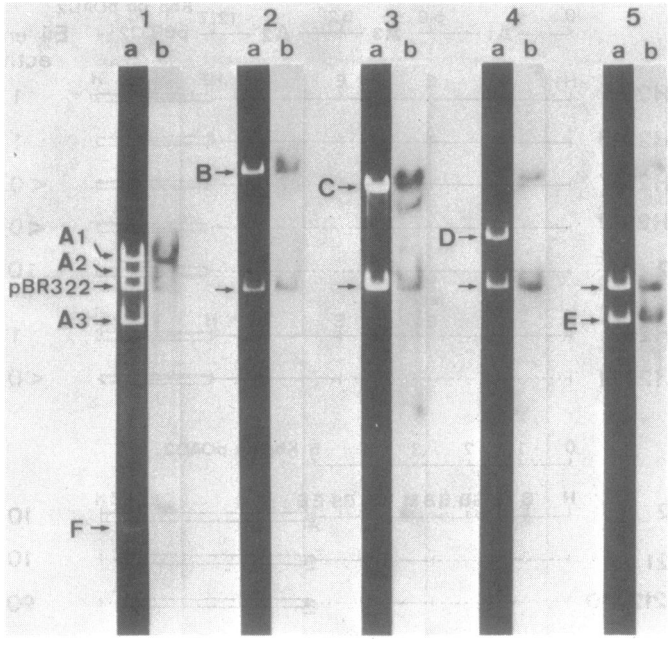


FIG. 3. Identification of restriction fragments which might contain a nucleotide sequence analogous to the EII structural gene. Plasmids pNDH28 (a hybrid plasmid consisting of pBR322 and the *Hin*dIII-A and -F fragments of pOAD2) (slot 1), pNDH6 (pBR322+B fragment) (slot 2), pNDH5 (pBR322+C fragment) (slot 3), pNDH3 (pBR322+D fragment) (slot 4), and pNDH10 (pBR322+E fragment) (slot 5) were digested with *Hin*dIII (slots 2 to 5) or *Hin*dIII+*Eco*RI (slot 1) and subjected to agarose (1%) gel electrophoresis. a, Ethidium bromide staining; b, Southern blot of the gel hybridized with ^{32}P-labeled pNDH2912.

+ fragment E) were digested with combinations of restriction enzymes followed by Southern blotting hybridization. A 3.56-kbp *Hin*dIII-*Eco*RI fragment and a 0.75-kbp (*Hin*dIII-*Bam*HI fragment were detected among the pNDH5 fragments (Fig. 4), indicating that the DNA region homologous to the A1 fragment is located between 37.1 (*Bam*HI site) and 37.9 (*Hin*dIII site) kbp (designated as the δ region). Similarly, the DNA region between 13.5 (*Hin*dIII site) and 16.1 (*Sma*I site) kbp (designated as the α region) contains a sequence analogous to the A1 region, evidenced by hybridization of both 1.4-kbp *Sma*I and 1.25-kbp *Hin*dIII-*Sma*I fragments with the A1 fragment (Fig. 4). Among *Bgl*II fragments of the pOAD2-*Hin*dIII-B fragment, the 9.2-kbp fragment (19.9- to 28.8-kbp region) and the 2.1-kbp fragment (16.8- to 18.9-kbp region) were detected with pNDH2912 as the probe, but the 1.0-kbp fragment (18.9 to 19.9 kbp) was not. *Pst*I digestion of fragment B gives double bands of 0.56 kbp in size which strongly hybridize with the probe and 9.4- and 1.4-kbp fragments which weakly hybridize. These results indicate that DNA regions homologous to the probe are present at both 16.8 to 17.4 kbp (the β region) and 28.2 to 28.8 kbp (the γ region).

Similar hybridization experiments were carried out on the DNA fragments derived from plasmids pOAD1 and pOAD3 and chromosomal DNA of KI72, using pNDH2912 as the probe, but no DNA regions homologous to the A1 fragment were detected (data not shown).

Identification of repeated sequences. The above results showed that the A1 fragment, on which the EII structural gene is located, hybridized at four regions (α, β, γ, and δ) on pOAD2. To examine the homology among these sequences, pNDH2912 digested with *Hin*dIII+*Bam*HI was hybridized with two different probes (Fig. 5). With pNDH513 (a hybrid plasmid consisting of pBR322, 28.8- to 29.8-kbp, and 37.1- to 37.9-kbp [δ] regions) as the probe, only one *Hin*dIII-*Bam*HI fragment, mapped between 0 and 0.77 kbp on pOAD2, was detected. Since pNDH501, consisting of pBR322 and the 28.8- to 30.8-kbp region, did not hybridize with the A1 fragments (see Fig. 1) with the same hybridization conditions (hybridization temperature, 65°C), we concluded that the 0.77-kbp fragment (0- to 0.77-kbp region) and the 0.71-kbp fragment (37.1- to 37.9-kbp region) contain the same homologous DNA sequence (designated RS-I). With pNDH10 containing the α region as the probe, in addition to the same 0.77-kbp fragment, two more fragments (0.62 and 1.58 kbp), which are located at both sides of the 70-bp *Bam*HI fragment (2.7- to 2.8-kbp region) on the

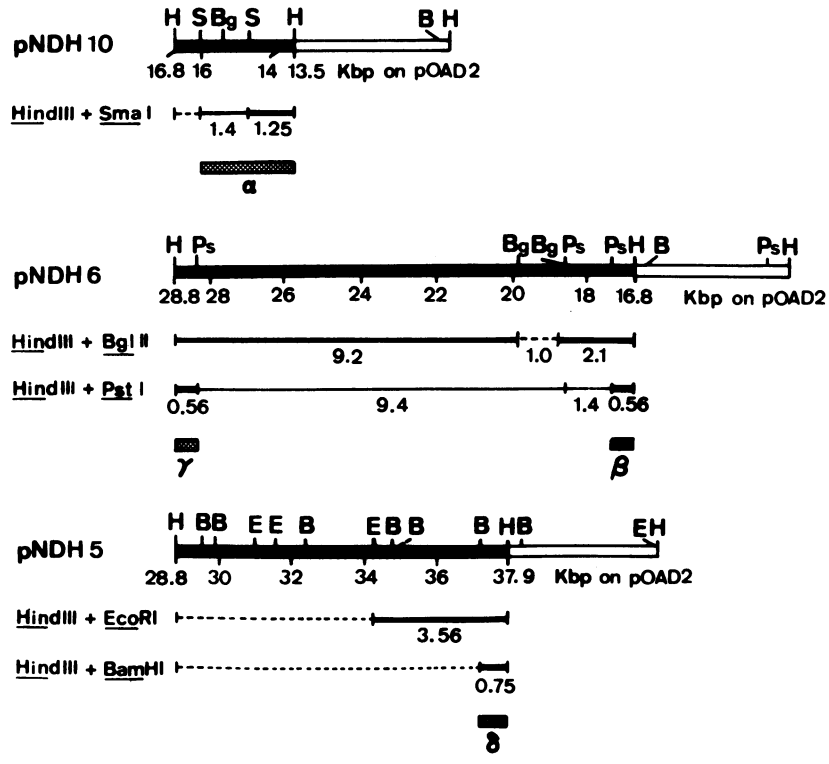


FIG. 4. Restriction fragments which hybridized to pNDH2912 containing the EII structural gene. The bold solid line, fine solid line, and broken line represent strongly hybridized, weakly hybridized, and nonhybridized fragments, respectively. The numbers under each line indicate the fragment size in kilobase pairs. Open boxes show pBR322 regions. Inserted fragments shown by heavy boxes are fragments E (pNDH10), B (pNDH6) and C (pNDH5). B, E, H, and S represent the restriction sites given in the legend to Fig. 2. Ps and Bg represent the *Pst*I and *Bgl*II sites.

map and are located between 2.1 and 4.2 kbp, were detected. These results indicate that at least two different repeated sequences exist on the A1 fragment (and also on the α region): one (RS-I) is recognized by both probes, and the other (designated RS-II) is located between 2.1 and 4.2 kbp and is recognized only by pNDH10.

(i) **RS-I.** Hybridization patterns of the pOAD2-B fragment digested with *Hin*dIII+*Bgl*II (and also *Hin*dIII+*Pst*I) are the same with both the pNDH2912 probe and the pNDH513 probe, indicating that the RS-I sequence is encoded at the β and the γ regions (Fig. 4 and 6). On the other hand, pNDH513 hybridized with the 1.25-kbp *Hin*dIII-*Sma*I fragment of pNDH10 (13.5 to 14.7 kbp), but not with the 1.4-kbp *Sma*I fragment of pNDH10 (14.7 to 16.1 kbp), which was recognized by pNDH2912 (Fig. 7B). No hybridization of A2, A3, C (except for the 28.8- to 29.8-kbp region and the δ region), D, or F fragments with pNDH513 as the probe were detected. From these results, we concluded that RS-I sequences are located at least in five regions: 0 to 0.77 (RS-I_A), 13.5 to 14.7 (RS-I_B), 16.8 to 17.4 (RS-I_C), 28.2 to 28.8 (RS-I_D), and 37.1 to 37.9 (RS-I_E) kbp.

(ii) **RS-II.** The fine loci of the RS-II regions on both A1 (RS-II_A) and E (RS-II_B) fragments were further studied by Southern hybridization experiments. Figure 8 shows the restriction fragments derived from pNDH2912 or pNL21 which hybridized with pNDH10. A 0.61-kbp *Pvu*II-*Mul*I fragment (3.2 to 3.8 kbp) which was mapped completely within the EII structural gene hybridized with the probe, suggesting that a homologous DNA sequence is contained in both RS-II regions. The right end of RS-II_A may be located between the *Pvu*II site at 3.8 kbp and the *Pst*I site at 4.2 kbp, because the probe hybridized with the 0.52-kbp *Pvu*II-*Bam*HI fragment but not with the 0.91-kbp *Pst*I-*Eco*RI fragment of pNL213. The left end of the repeated sequence might be present in the 0.62-kbp *Bam*HI fragment, since this fragment hybridized slightly with the probe (Fig. 8).

The location of the RS-II_B region on E fragments was analyzed by using a *Bam*HI 1.58-kbp fragment of pNL21 as the probe. The RS-II_B

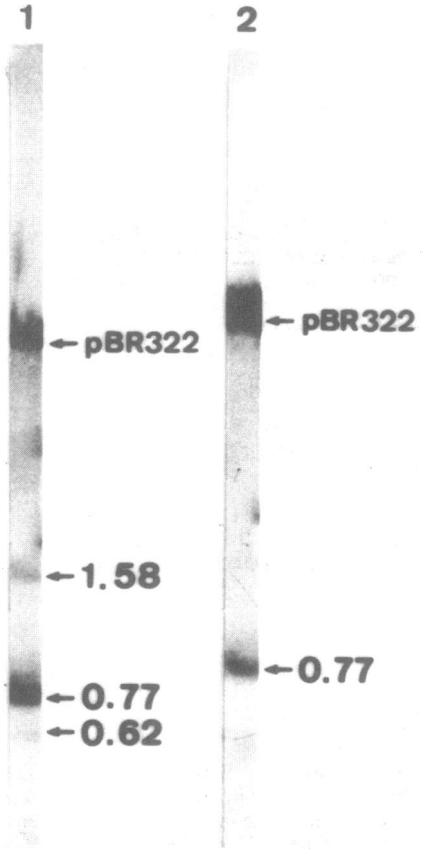


FIG. 5. Southern blot hybridization of pNDH2912 digested with *Hin*dIII+*Bam*HI. Slot 1, Southern blot of the gel hybridized with ^{32}P-labeled pNDH10 containing both RS-I and RS-II sequences. Slot 2, Southern blot of the gel hybridized with ^{32}P-labeled pNDH513 (derived from pNDH5 by partial digestion with *Bam*HI and ligation), which codes the RS-I sequence.

region lies within the 1.4-kbp *Sma*I fragment (14.7 to 16.1 kbp; Fig. 7B). Figure 9 shows the fine restriction map of RS-II$_A$ and RS-II$_B$. The restriction sites shown by stars are conserved in both sequences. These results suggest that these two sequences are homologous at least in the 1.1-kbp length between the *Bam*HI(b) site and the *Pvu*II site. Figure 10 summarizes the location of the repeated sequences and the genes for EI and EII in plasmid pOAD2.

DISCUSSION

Studies on the specific enzymes active on synthetic compounds provide a suitable system for examining how microorganisms evolve metabolic activity toward new compounds. There are two mechanisms by which an unnatural synthetic compound such as 6-aminohexanoic acid cyclic dimer might be enzymatically degraded: it might be hydrolyzed as an analog of a physiological substrate, or it might be decomposed by a newly evolved enzyme. The previous finding that EI and EII enzymes are active toward synthetic compounds but not toward any of the natural substrates tested suggests the latter possibility (9, 10).

The conclusion that two copies of the RS-II sequence are located on plasmid pOAD2 was also supported by our preliminary DNA sequencing of both RS-II regions. That only the one mapped in the region between 2.1 and 4.2 kbp (RS-II$_A$) produced functional EII protein suggests that the RS-II sequence located on the *Hin*dIII-E fragment (RS-II$_B$) might be translocated to the RS-II$_A$ locus to create a new enzyme, although no direct evidence of the translocation has been obtained. This hypothesis is also supported by the preliminary observation that insertion of the *lac*UV5 promoter fragment into pNDH10, which encodes RS-II$_B$, at the

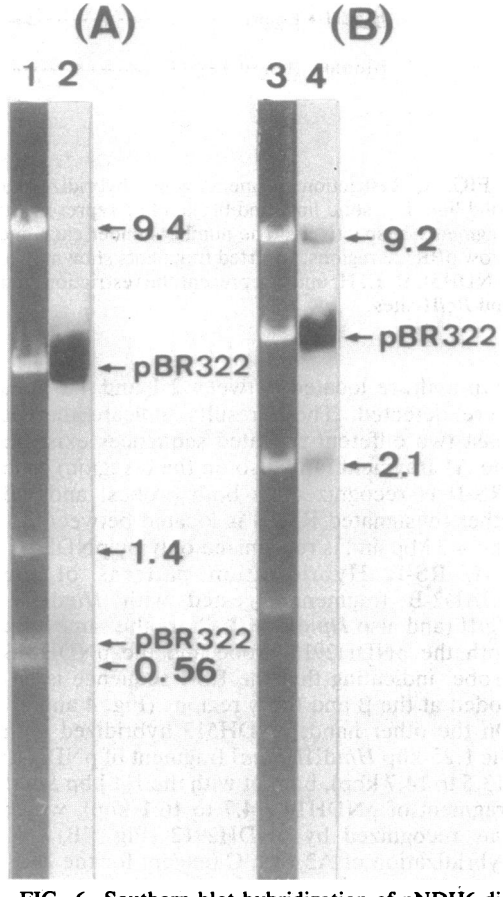


FIG. 6. Southern blot hybridization of pNDH6 digested with *Hin*dIII+*Pst*I (A) or *Hin*dIII+*Bgl*II (B). Slots 1 and 3, Ethidium bromide staining; slots 2 and 4, Southern blot of the gel hybridized with ^{32}P-labeled pNDH513.

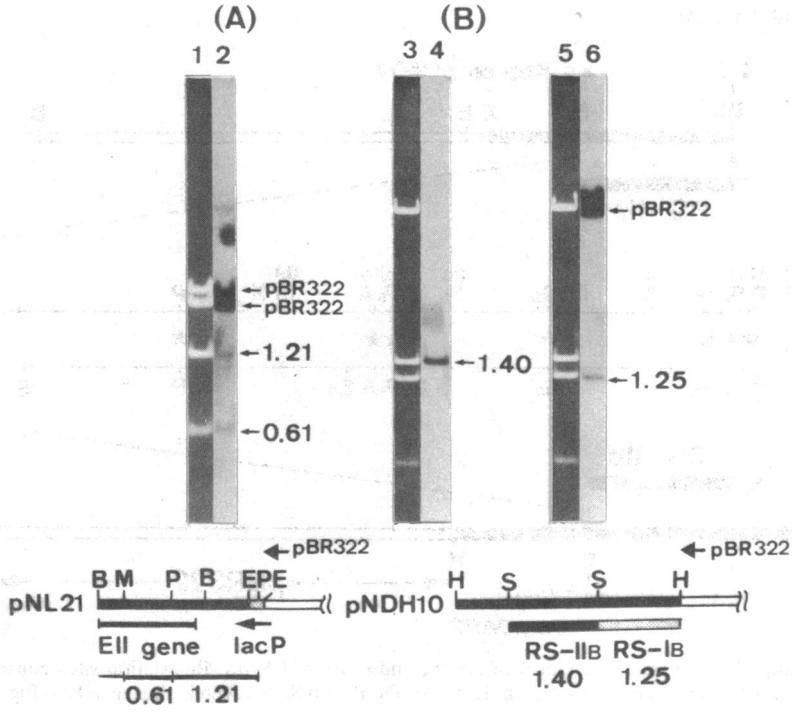


FIG. 7. Southern blot hybridization of pNL21 digested with *Pvu*II+*Mlu*I (A) or pNDH10 digested with *Hin*dIII+*Sma*I (B). Slots 1, 3, and 5, Ethidium bromide staining; slots 2, 4, and 6, Southern blot of the gel hybridized with ^{32}P-labeled pNDH10 (slot 2), the *Bam*HI 1.58-kbp fragment of pNL21 (slot 4), or pNDH513 (slot 6). B, M, E, H, and S represent the restriction sites given in the legends to Fig. 2 and 4. P indicates the *Pvu*II site.

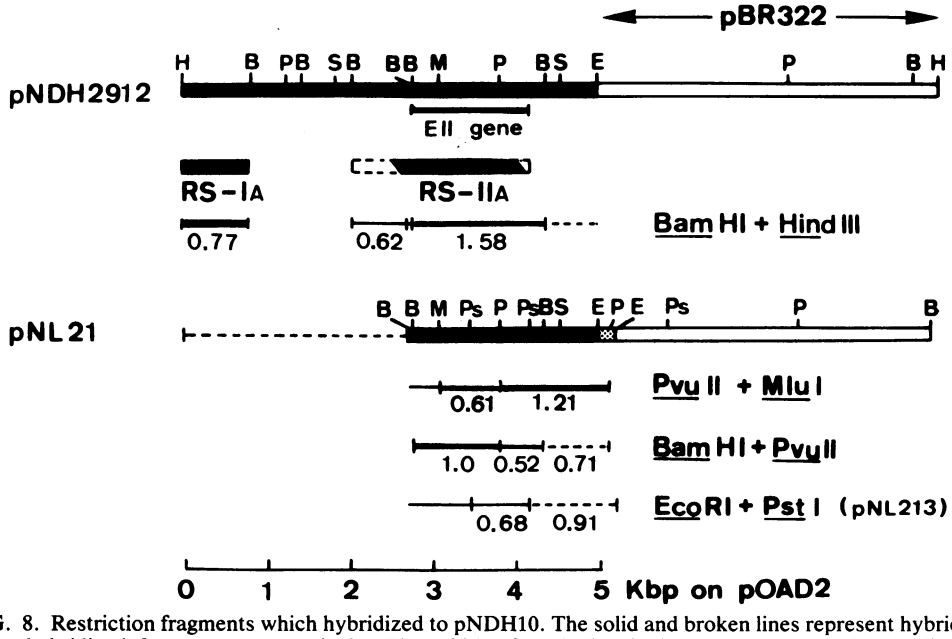


FIG. 8. Restriction fragments which hybridized to pNDH10. The solid and broken lines represent hybridized and nonhybridized fragments, respectively. The width of each line indicates the extent of hybridization. Numbers under the line show the fragment size in kilobase pairs. The locus of the EII structural gene is from 2.7 (*Bam*HI site) to 4.1 kbp on the A1 fragment. Since the 0.71-kbp *Eco*RI-*Pst*I fragment of pNL21 could not be separated from the fragment derived from the pBR322 region on agarose gel, plasmid pNL213, which lacks the left *Eco*RI site of pNL21, was used in the case of double digestion with *Eco*RI+*Pst*I. B, E, H, M, P, Ps, and S are the restriction sites given in the legends to Fig. 2, 4, and 7.

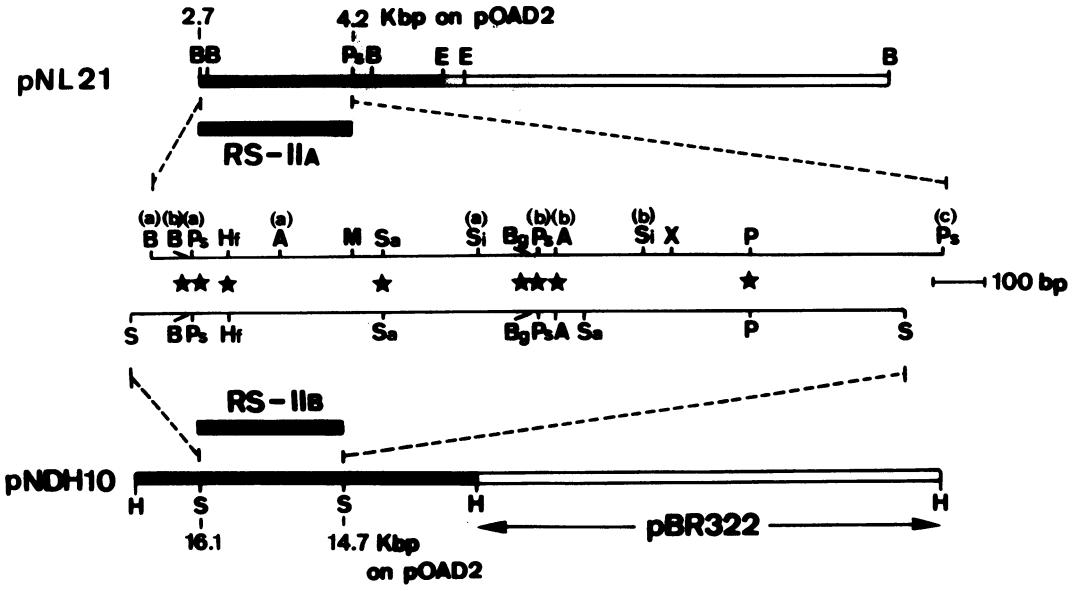


FIG. 9. Comparison of restriction sites of RS-II_A and those of RS-II_B. Restriction sites conserved between the two sequences are shown by stars. B, Bg, E, H, M, Ps, P, and S are given in the legends to Fig. 2, 4, and 7. A, Hf, Sa, Si, and X represent the *Ava*I, *Hin*fI, *Sal*I, *Sin*I, and *Xho*I sites, respectively.

*Sma*I site at 14.7 kbp on the pOAD2 map produced an EII-antigenic protein. After the duplication and translocation of RS-II on the plasmid, genetic changes such as mutations and recombination must have occurred, because the restriction patterns of the two RS-II regions are essentially different. For example, the *Ava*I(a), *Mlu*I, *Sin*I(a and b), and *Xho* I sites located in the EII gene are absent from the RS-II_B region (Fig. 9).

RS-I, which appeared five times on pOAD2, might be responsible for the rearrangement of the plasmid in the same way that the insertion sequence and transposon elements are (4). One

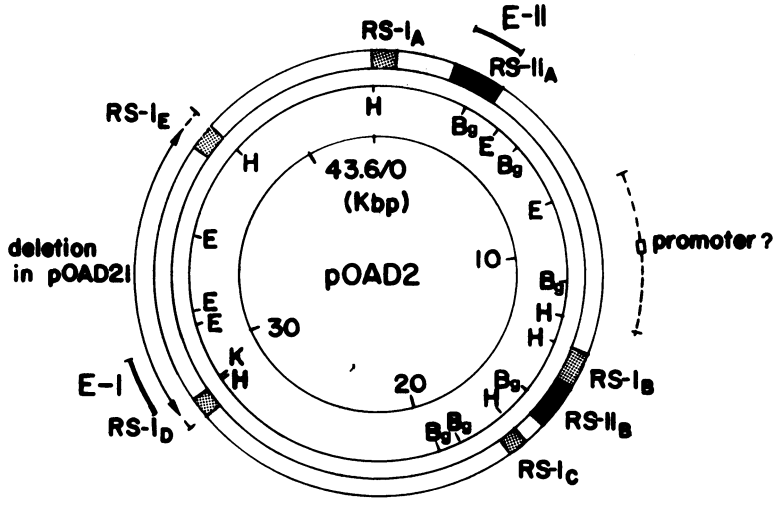


FIG. 10. Physical and function map of pOAD2. The EI gene (28.8 to 30.8 kbp), the EII structural gene (2.7 to 4.1 kbp), and the promoter site controlling the EII structural gene are recorded. Two repeated sequences, RS-I (shaded bar) and RS-II (solid bar), appeared five times and twice, respectively, and are distinguished by the following suffixes: A, B, C, D, and E for RS-I, and A and B for RS-II. The deleted region of 9.1 kbp in pOAD21 (28.0 to 38.0 kbp) is shown by an arrow. H, Bg, and E are the restriction sites given in the legends to Fig. 2 and 4. K is the *Kpn*I site.

piece of evidence for such activity of RS-I was obtained in the deletion plasmid pOAD21 from pOAD2, in which the deletion termini are located in or near the RS-I_D and RS-I_E regions (Fig. 10). This suggests that the translocation of RS-II might be helped by the RS-I regions.

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