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The nylon oligomer biodegradation system of *Flavobacterium* and *Pseudomonas*

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Abstract

This review article is a compendium of the available information on the degradation of a man-made compound, 6-aminohexanoate-oligomer, in *Flavobacterium* and *Pseudomonas* strains, and discusses the molecular basis for adaptation of microorganisms toward these xenobiotic compounds. Three plasmid-encoded enzymes, 6-aminohexanoate-cyclic-dimer hydrolase (EI), 6-aminohexanoate-dimer hydrolase (EII), and endo-type 6-aminohexanoate-oligomer hydrolase (EIII) are responsible for the degradation of the oligomers. Two repeated sequences, designated RS-I and RS-II, are found on plasmid pOAD2, which is involved in 6-aminohexanoate degradation in *Flavobacterium*. RS-I appears 5 times on the pOAD2, and all copies have the same sequences as insertion sequence IS*6100*. RS-II appears twice on the plasmid. RS-IIA contains the gene encoding EII, while RS-IIB contains the gene for the analogous EII′ protein. Both EII and EII′ are polypeptides of 392 amino acids, which differ by 46 amino acid residues. The specific activity of the EII enzyme is 200-fold higher than that of EII′. Construction of various hybrid genes demonstrated that only the combination of two amino acid residues in the EII′ enzyme can enhance the activity of the EII′ to the same level as that of EII enzyme.

Abbreviations: EI – 6-aminohexanoate-cyclic-dimer hydrolase; EII – 6-aminohexanoate-dimer hydrolase; EIII – endo-type 6-aminohexanoate-oligomer hydrolase; F-EI – EI from *Flavobacterium*; F-EII – EII from *Flavobacterium*; P-EI – EI from *Pseudomonas*; P-EII – EII from *Pseudomonas*; EII′ – a protein having 88% homology to the EII encoded on the RS-IIB region of pOAD2; *nylA* – gene for the EI enzyme; *nylB* – gene for the EII enzyme; *nylC* – gene for the EIII enzyme; *nylB′* – gene for the EII′ protein; kb – kilo-base-pairs

Introduction

Since the mid century, the rapid progress of the chemical industry has led to the distribution of a wide variety of synthetic compounds, both industrial products and wastes, into the environment. Recently, biodegradation or biological detoxification of xenobiotic compounds has been recognized as a useful way to eliminate such environmental pollutants. However, the efficiency of removal is highly dependent on the specific enzymes which can catalyze the desired degradation reactions.

Nylon-6 is produced from ∈-caprolactam by ring cleavage polymerization. It consists of more than 100 units of 6-aminohexanoic acid. During the polymerization reaction, some molecules fail to polymerize and remain to be oligomers, while others undergo head-to-tail condensation to form cyclic oligomers. These nylon oligomers are by-products from nylon factories, thereby contributing to the production of industrial waste material that could enter the environment.

Due to the high demand of biodegrading environmental pollutants, it is of interest to find bacterial strains that can survive within an environment

Table 1. Characteristics of nylon oligomer degradation enzymes.

Characteristics	Enzymes from *Flavobacterium* sp. KI72				Enzymes from *Pseudomonas* sp. NK87	
	F-EI	F-EII	EIII	EII′	P-EI	P-EII
M_r of subunit	52K	42K	37K	42K	52K	42K
No. of amino acids	(493)	(392)	(355)	(392)	(493)	(396)
Immuno-reactivity with						
anti-F-EI serum	+	-	-	-	+	-
anti-F-EII serum	-	+	-	+ (spur)	-	-
Optimum pH	7.4	9.0	7.0	NT	NT	8.0
Optimum temp.	34° C	40° C	42° C	NT	NT	48° C
Gene	F-*nylA*	F-*nylB*	*nylC*	*nylB′*	P-*nylA*	P-*nylB*
Encoded on	pOAD2	pOAD2	pOAD2	pOAD2	pNAD2	pNAD6

NT indicates not tested.

Table 2. Specific activity of F-EI, F-EII and EIII enzymes toward various nylon oligomers.

Substrate	Specific activity (mol NH_2 liberated/mol·s)		
	F-EI	F-EII	EIII
6-Aminohexanoate-			
linear-dimer	$< 1.0 \times 10^{-3}$	0.66	2.7×10^{-4}
linear-trimer	$< 1.0 \times 10^{-3}$	0.53	0.067
linear-tetramer	$< 1.0 \times 10^{-3}$	0.40	0.26
linear-pentamer	$< 1.0 \times 10^{-3}$	0.17	0.29
cyclic-dimer	2.4	$< 1.0 \times 10^{-4}$	$< 1.0 \times 10^{-4}$
cyclic-tetramer	$< 1.0 \times 10^{-3}$	$< 1.0 \times 10^{-4}$	0.22
∈-Caprolactam	$< 1.0 \times 10^{-3}$	$< 1.0 \times 10^{-4}$	$< 1.0 \times 10^{-4}$

with the cyclic dimer as the sole carbon and nitrogen source. It was found that *Flavobacterium* sp. KI72 and *Pseudomonas* sp. NK87 are such bacterial strains (Kinoshita et al. 1975; Kanagawa et al. 1989). The question was raised then as to how these microorganisms have evolved specific enzymes which can degrade such xenobiotic compounds. This review is a compendium of information on the degradation of nylon oligomers encoded on plasmids in *Flavobacterium* and *Pseudomonas* strains, and also discusses the molecular basis of adaptation toward xenobiotic compounds.

Nylon-oligomer degrading enzymes

Biochemical studies revealed three enzymes that are responsible for the degradation of 6-aminohexanoate oligomers, namely, 6-aminohexanoate-cyclic-dimer hydrolase (F-EI for *Flavobacterium* sp. KI72, P-EI for *Pseudomonas* sp. NK87) (Kinoshita et al. 1977; Kanagawa et al. 1988; Tsuchiya et al. 1989), 6-aminohexanoate-dimer hydrolase (F-EII for *Flavobacterium* sp. KI72, P-EII for *Pseudomonas* sp. NK87) (Kinoshita et al. 1981; Kanagawa et al. 1993), and endo-type 6-aminohexanoate oligomer hydrolase (EIII) (Negoro et al. 1992; Kakudo et al. 1993). The characteristics of the purified enzymes stated above are listed in Tables 1 and 2.

F-EI and P-EI are homodimer enzymes with subunits of molecular weight 52,000. The EI enzymes were active only toward the cyclic dimer but not toward more than 100 different natural compounds with amide bonds.

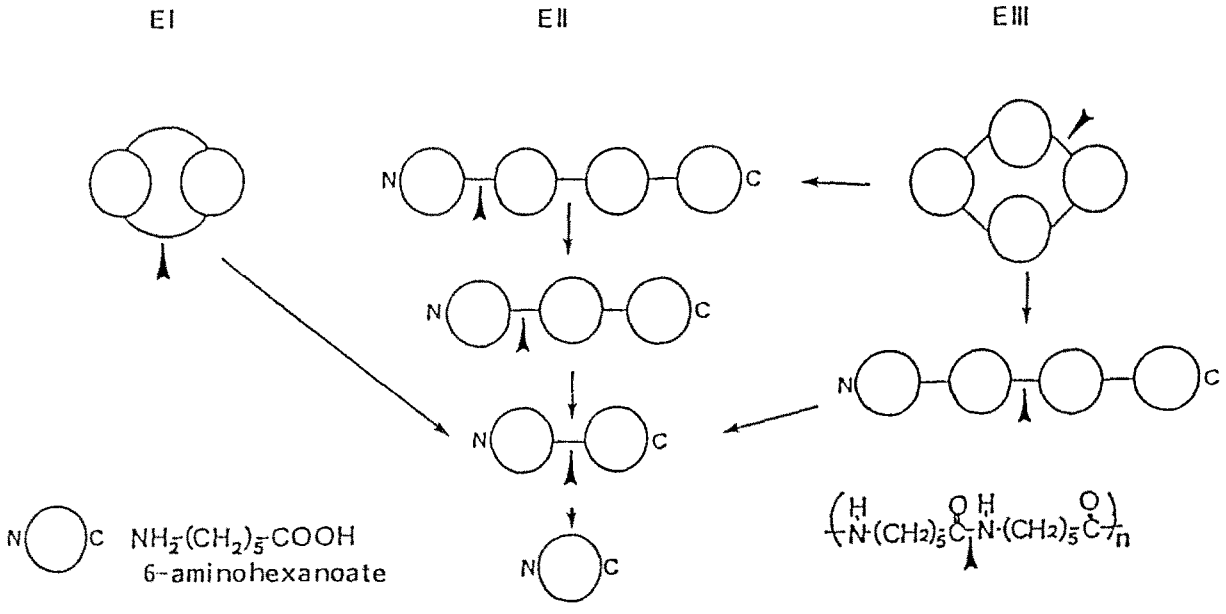

Fig. 1. Degradation of the nylon oligomers by 6-aminohexanoate-cyclic-dimer hydrolase (EI), 6-aminohexanoate-dimer hydrolase (EII) and endotype 6-aminohexanoate-oligomer hydrolase (EIII).

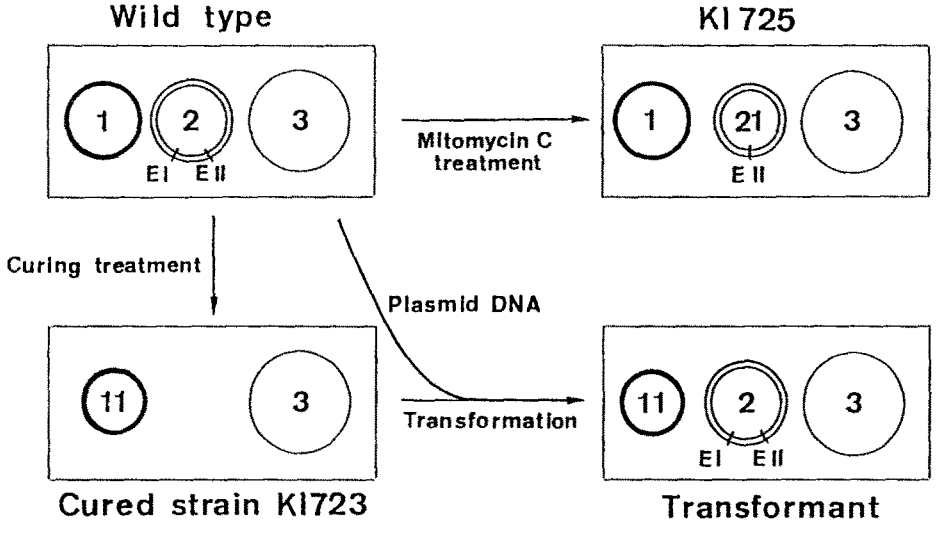

Fig. 2. Genetic construction of cured and transformant strains. Strain KI725, which grows on the 6-aminohexanoate-linear dimer but not on the cyclic dimer, was obtained by cultivation of KI72 on LB medium containing mitomycin C (0.5 μg/ml). KI725 possessed a plasmid pOAD21, in which a 9-kb region flanked by RS-I_D and RS-I_E sequences in pOAD2 was deleted (See Fig. 4).

F-EII and P-EII were also dimeric enzymes with two homologous subunits of molecular weight 42,000. These enzymes were active on 6-aminohexanoate oligomers ranging from dimer to hexamer but not on icosamer and hectamer. In addition, no activity was detected when these enzymes were tested with more than 100 different natural compounds possessing amide bonds. Furthermore, it was shown that the active site in the F-EII protein involves a serine residue (Ser112) (Negoro et al. 1989).

The EIII enzyme was either a homodimer or a trimer with subunits having a molecular weight of

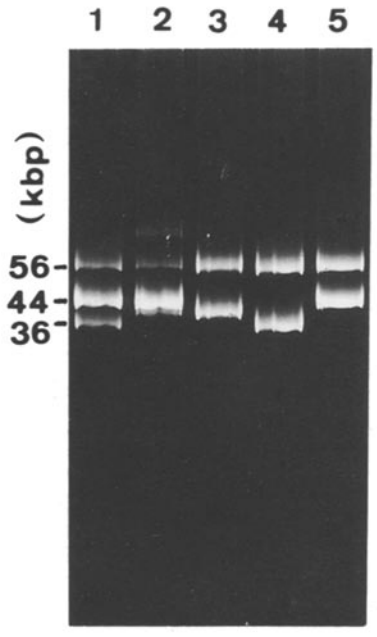

Fig. 3. Agarose gel electrophoresis of plasmid DNA. Plasmids obtained by CsCl-ethidium bromide density gradient centrifugation were fractionated by agarose gel (0.55%) electrophoresis. Slot 1, KI723T1; slot 2, KI72; slot 3, KI722; slot 4, KI723; slot 5, KI724.

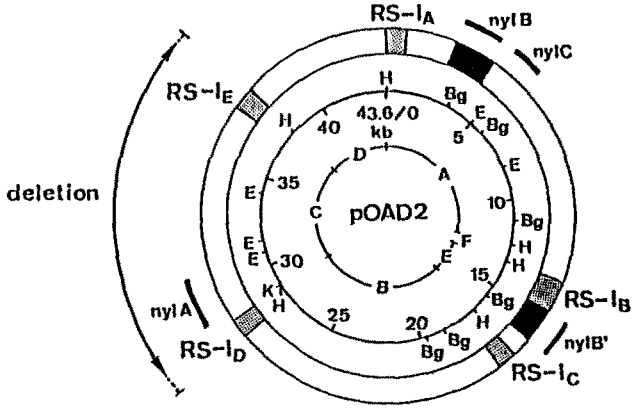

Fig. 4. Structural and functional map of plasmid pOAD2. The nylon oligomer degradation genes are at the following loci: the F-*nylA* (29.1–30.6 kb region on pOAD2 map), F-*nylB* (2.7–4.1 bp region), *nylB'* (14.7–16.1 kb region), and *nylC* (4.2–5.2 kb region). RS-I and RS-II are repeated sequences identified by Southern hybridization experiments. RS-I appeared 5 times on pOAD2. The F-*nylB* and *nylB'* genes are located in RS-II$_A$ and RS-II$_B$ regions, respectively. Bg, E, H, and K represent restriction sites for *Bgl*II, *Eco*RI, *Hind*III, and *Kpn*I, respectively. The positions of six *Hind*III fragments (A to F) of pOAD2 are shown in the innermost circle. pOAD21 was obtained from pOAD2 by homologous recombination between the direct repeats RS-I$_D$ and RS-I$_E$. The arrow indicates the region deleted in pOAD2.

37,000. It was active on the cyclic tetramer and pentamer and on linear oligomers higher than trimer. EIII was not active on the amide bonds of natural compounds tested so far.

From the gathered experimental results, it was concluded that the degradation of various nylon oligomers follows the pathway shown in Fig. 1.

Plasmid dependence of nylon oligomer degradation enzymes

Genes responsible for the metabolisms of organic compounds are often encoded on degradative plasmids (Frantz & Chakrabarty 1986). The nylon oligomer degradation genes in *Flavobacterium* and *Pseudomonas* were confirmed to be plasmid-coded by curing, transformation and cloning experiments. Treatment with curing agents such as mitomycin C, ethidium bromide and sodium dodecyl sulfate eliminated the metabolic activity (Negoro et al. 1980) (Fig. 2). After treatment, more than 80% of the clones had lost the metabolic activities toward both the cyclic and linear dimer. No reverse mutation was observed in more than 10^{10} cells of the cured strains tested. Moreover, it was found that the metabolic activities can be simply restored by transforming the cured strain with plasmid DNA of the wild strain (Fig. 2). These results, in addition with immunological evidence (Negoro et al. 1980), indicate that the inability of the cured strain to grow on the cyclic dimer and linear dimer is due to the absence of the EI and EII enzymes.

Flavobacterium sp. KI72 possessed three kinds of plasmids, pOAD1 [39 kilo-base-pairs (kb)], pOAD2 (44-kb) and pOAD3 (56-kb). The molecular size of the plasmids harbored in the wild-type, the cured and the transformant strains was determined by electron microscopy (data not shown) and agarose gel electrophoresis (Fig. 3). Curing readily eliminates pOAD2 with the loss of the EI and EII enzyme activities. On the other hand, pOAD1 either remained intact (KI722), or was only partially deleted (KI723) even after curing. Transformation of *Flavobacterium* sp. KI723 with plasmid DNA of the wild-type strain showed that KI723 readily accepted pOAD2 and simultaneously recovered its EI and EII enzyme activities (Fig. 2). These results demonstrate that the EI and EII enzymes were encoded on plasmid pOAD2 (Negoro et al. 1980) (Fig. 4). It was also shown that EIII enzyme activity was detected in the wild type KI72 and transformant

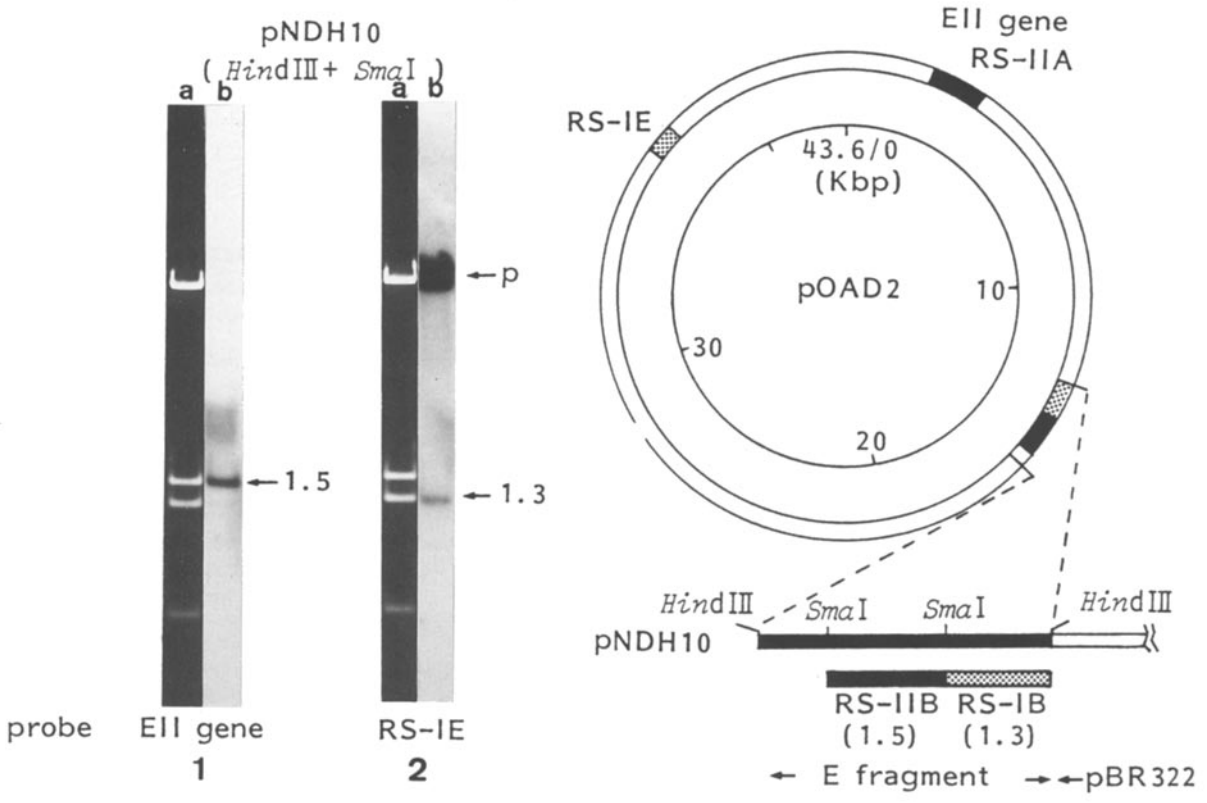

Fig. 5. Southern blot hybridization of pNDH10 digested with *Hind*III + *Sma*I. Slot a, ethidium bromide staining; slot b, Southern blot of the gel hybridized with ^{32}P-labeled *Bam*HI fragment of pNL21 (RS-II$_A$ probe) (slot 1), or pNDH513 (RS-I$_E$ probe) (slot 2).

KI723T1 but not in the cured strain KI723 (Negoro et al. 1992).

Plasmid pOAD2 is cleaved by *Hind*III into 6 fragments (A, B, C, D, E and F; Fig. 4). The F-*nylA* gene on the C fragment and the F-*nylB* and *nylC* gene on the A fragment were inserted into pBR322 and transformed to *E. coli*. Transformants containing hybrid plasmids encoding the A fragment produced the EII and EIII enzymes, and those encoding the C fragment produced the EI enzyme. Subcloning of smaller fragments coding the F-*nylA*, F-*nylB* or *nylC* genes revealed the precise location of these genes on pOAD2 (Fig. 4).

Pseudomonas sp. NK87 harbors 6 different plasmids, namely, pNAD1 (20-kb), pNAD2 (23-kb), pNAD3 (51-kb), pNAD4 (57-kb) and pNAD6 (80-kb). Genes for P-EI and P-EII were cloned into *E. coli* using pUC12 as the cloning vector, and the corresponding enzymes were expressed in *E. coli*. Using the cloned genes as a probe for the Southern hybridization experiments, the P-*nylA* and P-*nylB* genes were found to be located on pNAD2 and pNAD6, respectively (Kanagawa et al. 1987).

Repeated sequence in pOAD2

The results of Southern hybridization tests using various fragments of pOAD2 indicated that there are at least two types of repeated sequences, RS-I and RS-II, in pOAD2 (Negoro et al. 1983). One of the results from the hybridization experiments is shown in Fig. 5. The repeated sequences appeared either 5 times (RS-I) or twice (RS-II) on the plasmid (Fig. 4). Sequencing studies revealed that the organization of the rpeated sequence is as follows:

RS-I

The five RS-I regions were identical over a length of 880-bp, except that the 420-bp region at the 3′ terminus of the 880-bp region was duplicated in RS-IB (Kato et al. 1993). In addition, the homology instantaneously ended at the borderline. Of the five RS-I regions, two (RS-ID and RS-IE) had a reverse orientation on the map of pOAD2 (Fig. 4). In the 880-bp region, a 765-bp open reading frame was observed to have 78-bp internal inverted repeat sequences. A homology search

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                          PvuII                                        60
EII   MNARSTGQHPARYPGAAAGEPTLDSWQEAPHNRWAFARLGELLPTAAVSRRDPATPAEPV
EII'    TPT  S      D             P        H   MV S      PVNA GHAL

                                                                      120
      VRLDALATRLPDLEQRLEETCTDAFLVLRGSEVLAEYYRAGFAPDDRHLLMSVSKSLCGT
      A  G I AQ         Q Y         T  V

                                          PstI  BglII                 180
      VVGALIDEGRIDPAQPVTEYVPELAGSVYDGPSVLQVLDMQISIDYNEDYVDPASEVQTH
           V

        NaeI               SstII                                      240
      DRSAGWRTRRDGDPADTYEFLTTLRGDGGTGEFQYCSANTDVLAWIVERVTGLRYVEALS
      G         H                S

                    SalI          Sau3AI                              300
      TYLWAKLDADRDATITVDQTGFGFANGGVSCTARDLARVGRMMLDGGVAPGGRVVSQGWV
                         T      H                                   ED

         NaeI                                                         360
      ESVLAGGSREAMTDEGFTSAFPEGSYTRQWWCTGNERGNVSGIGIHGQNLWLDPRTDSVI
      RR       H      K   NT  D                                  L

                         BamHI      392
      VKLSSWPDPDTRHWHGLQSGILLDVSRALDAV
                E   R  N
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Fig. 6. Amino acid sequences of the F-EII and EII′ proteins. The amino acid sequence of EII′ is identical to that of F-EII with the exception of the amino acids given below the F-EII sequence. The positions of the restriction sites used for the construction of the hybrid proteins are shown above the sequence.

of the DNA data base demonstrated that the 880-bp sequence was identical to the IS*6100* sequence found in *Mycobacterium fortuitum*. The presence of identical *IS* elements in *Flavobacterium* and *Mycobacterium* strains suggest that these elements are distributed widely among microorganisms, both among gram-negative and gram-positive bacteria. Similar sequences were also found in plasmid pNAD2 which contains the P-*nylA* gene of *Pseudomonas* sp. NK87, but not in the other 5 plasmids of the chromosome of strain NK87. The absence of sequence diversity in the *IS* elements indicates that the multiplication and distribution of *IS* elements occurred very recently. Coexistence of similar insertion sequences and *nylA* genes in the two nylon oligomer degradative plasmids, pOAD2 and pNAD2, may suggest that the IS sequence provides some growth advantages to the host strains to select the *nyl* gene.

RS-II

There are two RS-II regions, of which RS-IIA contains the F-*nylB* gene, while RS-IIB contains the analogous *nylB′* gene. The F-*nylB* and *nylB′* have open reading frames (ORF) of the same length (392 amino acids), the same Shine-Dalgarno sequence, and the same position of the initiation and termination codons. Of the 392 amino acid residues encoded by these ORFs, only 46 residues were different. The hypothetical protein encoded by the *nylB′* was named EII′. This indicates that the F-*nylB* and *nylB′* genes have evolved from the same ancestor gene. The EII′ had a very low catalytic activity (1/200 of EII activity) toward linear dimer (Okada et al. 1983). These results suggest that an ancestral F-*nylB* gene on pOAD2 was duplicated, and one of them was mutated to increase the catalytic activity 200 fold.

Construction of hybrids between F-*nylB* and *nylB′*

Though the F-EII and EII′ have the same number of amino acid residues and are similar in their amino acid sequence, their catalytic activities are quite different. In due accord, construction of hybrid enzymes was conducted for the following reasons: i) to evaluate the role of the amino acid alterations on the catalyt-

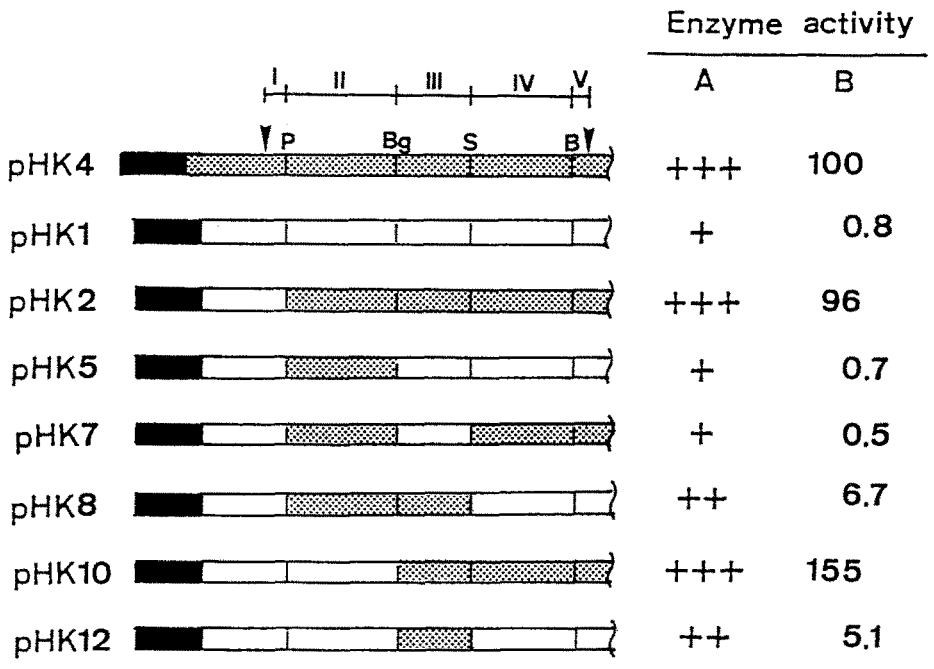

Fig. 7. Plasmid structures and enzyme activities of cell extracts of *E. coli* harboring hybrid plasmids. All hybrid plasmids contain the same vector DNA (a 2.3-kb *Hind*III-*Pvu*I fragment of pBR322). Arrows indicate the position of the initiation and termination codons of the F-*nylB* gene. ▨: DNA originated from F-*nylB* and its flanking region. □: DNA from *nylB'* and its flanking region. ■: *lac* promoter fragment. B, Bg, P and S indicate restriction sites for *Bam*HI, *Bgl*II, *Pvu*II and *Sal*I, respectively. (A) Enzyme activities in cell extracts of *E. coli* C600r$_K^-$m$_K^-$ harboring the pHK plasmid indicated toward 6-aminohexanoate dimer, as examined by paper chromatography. Approximately 50% of the substrate was hydrolyzed within 5 min (+++), 1 h (++), and 20 h (+) of the reaction time. (B) Activities in cell extracts of *E. coli* as measured by reverse-phase HPLC (C_{18} column), divided by the amount of antigenic F-EII protein in extracts, as measured by Rocket quantitative immunoelectrophoresis. The specific activities are defined as enzyme activity per amount of antigen and are expressed as a percentage of that of *E. coli* (pHK4).

ic activity; ii) to target amino acid residues useful for *in vitro* mutagenesis; iii) to know whether the hybrid enzyme will possess the characteristics of both parental enzymes.

Since restriction sites of *Pvu*II, *Bgl*II, *Sal*I, and *Bam*HI (respectively at 74, 483, 771, and 1,141 bp downstream from the initiation codon) are conserved in F-*nylB* and *nylB'*, exchanging DNA fragments at those restriction sites between the two genes is possible without changing the reading frame (Fig. 6). The numbers of the amino acids thus altered were 5 in the 74-bp fragment flanked by the initiation codon and the *Pvu*II site (region I for F-*nylB*, I' for *nylB'*), 23 in the 409-bp fragment flanked by the *Pvu*II and *Bgl*II sites (region II for F-*nylB*, II' for *nylB'*), 3 in the 288-bp fragment flanked by the *Bgl*II and *Sal*I sites (region III for F-*nylB*, III' for *nylB'*), and 15 in the 370-bp fragment flanked by the *Sal*I and the *Bam*HI sites (region IV for F-*nylB*, IV' for *nylB'*). There are no amino acid differences on the region downstream of the *Bam*HI site (region V). Comparison of the specific activities of the various hybrid proteins (Negoro et al. 1984; Kato et al. 1991; Fujiyama et al. 1991) revealed that:

1) The enzymes produced by *E. coli* harboring pHK4 (I-II-III-IV-V) and pHK2 (I'-II-III-IV-V) have almost the same specific activity (enzyme activity per amount of antigenic F-EII protein in cell extracts) (Fig. 7). This suggests that the five amino acid alterations in region I have no effect on the catalytic activity.
2) The specific activity directed by pHK2 decreased drastically to the EII' level upon replacing region III by region III' (see pHK7) (Fig. 7). This result is confirmed by pHK12 (I'-II'-III-IV'-V), which directed the production of a hybrid enzyme (Hyb-12) which had a specific activity 7 fold higher than EII'. Moreover, the intermediate level of the enzyme activity of the Hyb-12 hybrid enzyme was restored to the F-EII level by replacing region IV' with region IV (see pHK10, Hyb-10 protein).

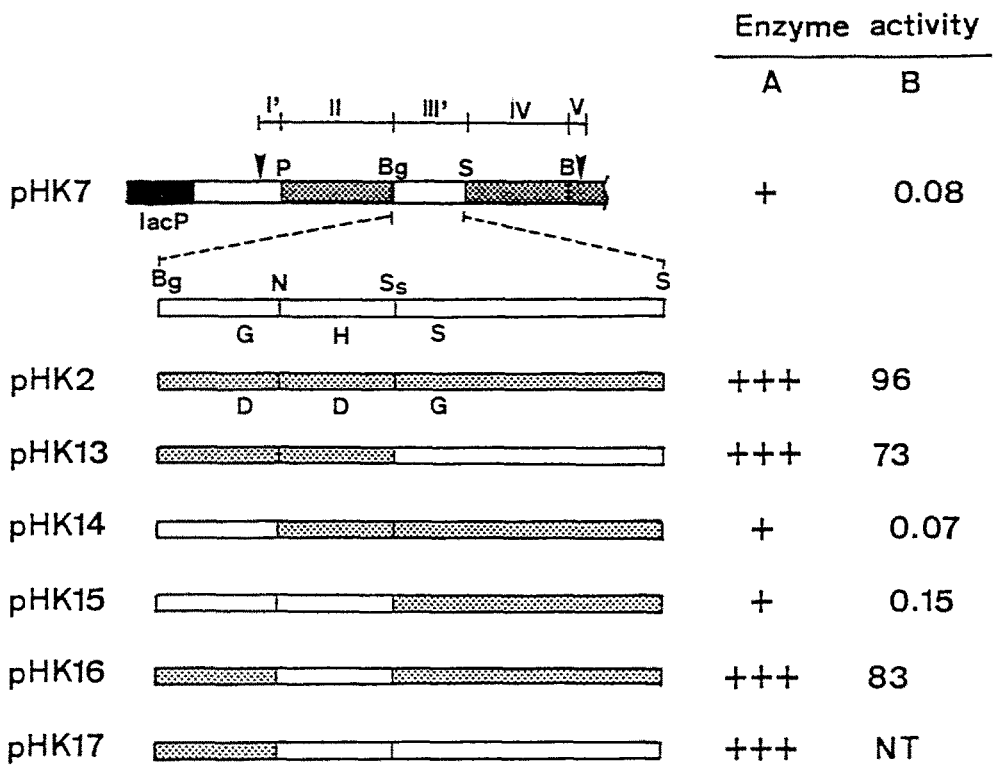

Fig. 8. Relationships between plasmid structure and enzyme activity. The structures of pHK13, 14, 15, 16, and 17 are identical to that of pHK7 except in the *BglII-SalI* region. The structures of pHK2 and pHK4 are identical except in the *lac* promoter-*PvuII* region. ▨: DNA originated from F-*nylB* and its flanking region. □: DNA from *nylB'* and its flanking region. ■: *lac* promoter fragment. Arrows indicate the position of the initiation and termination codon. Amino acid residues that differ between the F-EII and EII' are shown as one-letter codes under the map. N, and Ss indicate *NaeI* and *SstII* sites, respectively. The qualitative (A) and quantitative (B) enzyme assays, and abbreviations for the other restriction sites are given in the legend to Fig. 7. NT indicates 'not tested'. The activities were expressed as percentages of the activities per amount of antigen compared to that of *E. coli* (pHK4).

These results suggest that amino acid alterations occurring in region III' (162–257 regions in the amino acid sequences) are essential for increasing the enzyme activity, and that this effect is enhanced by amino acid alterations in the region IV' (the 258–380 region).

3) Among the three alterations found in region III, only position 181 is critical for its activity (Kato et al. 1991), based on the fact that alteration of Asp181 (F-EII) to Gly (EII') drastically decreased the activity to the EII' level (see pHK14) (Fig. 8).

4) The activity of Hyb-8 (I'-II-III-IV'-V) is about 2–7% of that of F-EII (see pHK8Δ11) (Fig. 9). The enzyme activities were restored to the EII level in cells harboring pHK21, which carries a one base substitution that replaces His266 by Asn in pHK8Δ11. These experiments lead to the conclusion that only the single amino acid alteration of His266 to Asn is essential among the 15 alterations in the region IV (Kato et al. 1991).

5) Integration of the two amino acid replacements at position 188 and 266 identified above into the *nylB'* gene led to an increase of the activity of EII' to the level of the parental F-EII enzyme (Fig. 9). The enzyme activity of the *E. coli* harboring pHK23 is almost the same as that of *E. coli* harboring pHK4. These results indicate that only two amino acid replacements in the EII' protein are involved in the increase of the activity back to the level of the parental F-EII enzyme. In addition, it was also shown that the remaining 44 amino acid substitutions do not affect enzyme activity, although these 44 alterations included many non-synonymous substitutions (Kato et al. 1991).

The results suggest that very few amino acid alterations are required for an enzyme to obtain a new activity such as during evolution of EII from its ancestor. Microor-

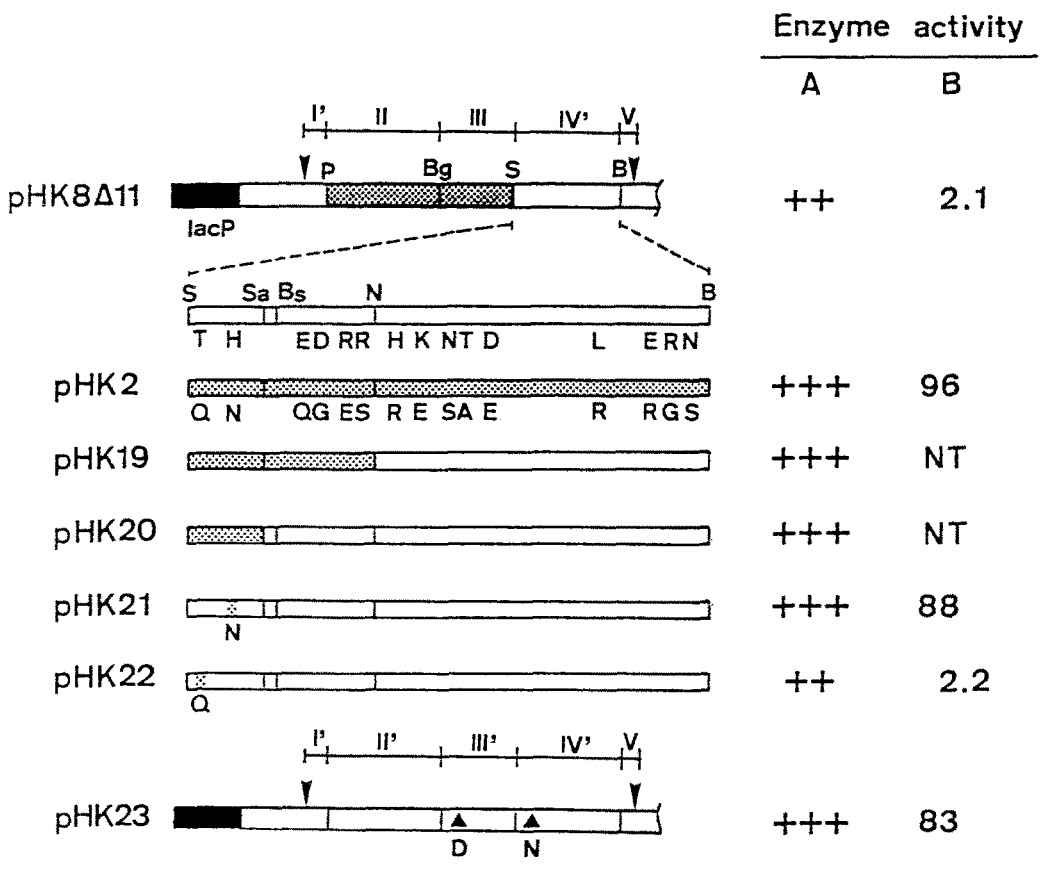

Fig. 9. Relationship between the plasmid structure and the enzyme activity. Symbols for the DNA regions, qualitative (A) and quantitative (B) enzyme assays, and abbreviations for restriction sites are given in the legends of Figs 7 and 8. Bs and Sa indicate restriction sites for *Bss*HII and *Sau*3AI, respectively. The activities are expressed as percentages of the activities per amount of antigen compared to that of *E. coli* (pHK4). NT indicates 'not tested'. The structures of pHK8Δ11, 19, 20, 21, and 22 are identical except in the *Sal*I-*Bam*HI region. Differences in amino acid residues between the F-EII and EII′ are shown as one-letter codes under the map. Plasmid pHK23 produces a protein that is identical to EII′ except that Gly181 and His266 were replaced by Asp and Asn, respectively.

ganisms may possess a cryptic gene such as EII′gene, and increasing its activity by mutations may lead to degradation of a xenobiotic compound.

Alteration of catalytic activity of nylon oligomer degradation enzyme by site-directed mutagenesis

Since the construction of the hybrid genes demonstrated that the Asp181 → Gly alteration in the F-EII enzyme drastically affects the catalytic function, the effect of amino acid alteration at this position was further examined. One of the hybrids, Hyb-2, produced by a plasmid pHK2, had 6-aminohexanoate-dimer hydrolyzing activity which is nearly equal to the activity of the F-EII enzyme, though region I of the F-EII is replaced by region I′ of the EII′ (Negoro et al. 1984). Residue Asp181 of the Hyb-2 protein was replaced by Asn, Glu, His, and Lys, using site-directed mutagenesis (Hatanaka et al. 1991) (Table 3). pHK2N, 2E, 2K and 2H produced enzymes in which Asp181 in the Hyb-2 was replaced with Asn (Hyb-2N), Glu (Hyb-2E), Lys (Hyb-2K), and His (Hyb-2H), respectively. The specific activities of Hyb-2N (Asn181) and Hyb-2E (Glu181) were 1/20 and 1/200 of those of the parental F-EII (Asp181) enzyme, respectively. Replacement to Lys or His at position 181 resulted in the complete loss of the activities (less than 1/5,000 of the F-EII enzyme) (Table 3).

To test whether the decrease in the EII activities is due to a decrease in k_{cat} values or an increase in

Table 3. Effects of amino acid alterations at position 181 on 6-aminohexanoate-dimer hydrolase activity.

Enzyme	Enzyme activity
F-EII	100
Hyb-2	96
Hyb-2N	5.0
Hyb-2E	0.42
Hyb-2K	< 0.02
Hyb-2H	< 0.02

Enzyme activities in cell extracts of *E. coli* harboring the pHK plasmid indicated were assayed by HPLC, and expressed as percentages of the activities per amount of antigen compared to the value of *E. coli* (pHK4). Hyb-2 is the hybrid enzyme produced by pHK2. Hyb-2N, -2E, -2K, and -2H are identical to Hyb-2, except that Asp181 (EII type) is changed to Asn, Glu, Lys, and His, respectively.

K_m values, the Hyb-2N and Hyb-2E enzymes were purified to homogeneity by three passages through a DEAE-Sephadex column (Hatanaka et al. 1991). Though Hyb-2N and Hyb-2E still had high k_{cat} values (approximately 30% of the k_{cat} of the F- EII (Asp181) enzyme), the K_m values were different compared to the wild-type enzyme: 80 mM (Asn181) and 220 mM (Glu181). These values were 4 times (Asn181) and 11 times the value (Glu181) of the F-EII enzyme. These results suggest that this mutation affects the binding of the substrate to the enzyme.

Site-directed mutagenesis is an effective tool for identifying the function of specific amino acids in enzymes that differ one or a few amino acid replacements. However, when related enzymes differ by more than two amino acids, as in the case of F-EII and EII′, the construction and characterization of hybrids can serve as an effective tool to examine the influence of specific amino acid alterations.

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