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MINI-REVIEW

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Biodegradation of nylon oligomers

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Abstract This mini-review is a compendium of the degradation of a man-made compound, 6-aminohexanoate-oligomer, in *Flavobacterium* strains. The results are summarized as follows:

1. Three enzymes, 6-aminohexanoate-cyclic-dimer hydrolase (EI), 6-aminohexanoate-dimer hydrolase (EII), and endotype 6-aminohexanoate-oligomer hydrolase (EIII) were responsible for degradation of the oligomers.
2. The genes coding these enzymes were located on pOAD2, one of three plasmids harbored in *Flavobacterium* sp. KI72, which comprised 45,519 bp.
3. The gene coding the EII′ protein (a protein having 88% homology with EII) and five IS*6100* elements were identified on pOAD2.
4. The specific activity of EII was 200-fold higher than that of EII′. However, altering two amino acid residues in the EII′ enzyme enhanced the activity of EII′ to the same level as that of the EII enzyme.
5. The deduced amino acid sequences from eight regions of pOAD2 had significant similarity with the sequences of gene products such as *oppA-F* (encoding oligopeptide permease), *ftsX* (filamentation temperature sensitivity), *penDE* (isopenicillin *N*-acyltransferase) and *rep* (plasmid replication).
6. The EI and EII genes of *Pseudomonas* sp. NK87 (another nylon oligomer-degrading bacterium) were also located on plasmids.
7. Through selective cultivation using nylon oligomers as a sole source of carbon and nitrogen, two strains which initially had no metabolic activity for nylon oligomers, *Flavobacterium* sp. KI725 and *Pseudomonas aeruginosa* PAO1, were given the ability to degrade xenobiotic compounds. A molecular basis for the adaptation of microorganisms toward xenobiotic compounds was described.

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Introduction

Since the middle of the twentieth century, rapid developments in the chemical industry have led to the distribution of a wide variety of synthetic compounds into the environment. Recently, biodegradation of xenobiotic compounds has been recognized as a useful way to eliminate environmental pollutants. However, the efficiency of removal is highly dependent on the specific enzymes that can catalyze the desired degradation reactions. In addition to the environmental point of view, microbial degradation of synthetic compounds provides a good system for studying how microorganisms acquire the ability to degrade such compounds.

Nylon-6 is produced from ε-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 as oligomers, while others undergo head-to-tail condensation to form cyclic oligomers. These nylon oligomers are by-products rejected by nylon factories, thereby contributing to the increase in industrial waste material entering the environment. Due to the high demand for biodegradable environmental pollutants, it is important to find bacterial strains that can degrade these compounds. We have isolated two microorganisms, *Flavobacterium* sp. KI72 (IFO14590) and *Pseudomonas* sp. NK87, which grow on the cyclic dimer of 6-aminohexanoate as a sole source of carbon and nitrogen, from soil and waste water from nylon factories, respectively (Kinoshita et al. 1975; Kanagawa et al. 1989). The KI72 and NK87 strains almost completely utilized 1% of the cyclic dimer within 24 h. *P. aeruginosa*, which degrades ε-caprolactam, was recently isolated (Kulkarni and

Kanekar 1998). Moreover, it is reported that nylon-6 and nylon-66 were degraded by a peroxidase of lignin-degrading fungi (Deguchi et al. 1998). The question was raised then as to how these microorganisms have evolved specific enzymes that can degrade such xenobiotic compounds.

In *Pseudomonas*, degradative pathways such as those for toluene, camphor, salicylates, alkanes, and naphthalene are dependent on degradative plasmids called TOL, CAM, SAL, OCT, and NAH plasmids, respectively (Frantz and Chakrabarty 1986). This mini-review describes the biodegradation of nylon oligomers by plasmids in *Flavobacterium* and *Pseudomonas*. In addition, the artificial expansion of metabolic activities to degrade the nylon oligomer and a molecular basis of the adaptation of microorganisms toward xenobiotic compounds are described.

Nylon oligomer-degrading enzymes

Biochemical studies revealed that three enzymes produced by *Flavobacterium* sp. KI72, 6-aminohexanoate-cyclic-dimer hydrolase (EI; Kinoshita et al. 1977), 6-aminohexanoate-dimer hydrolase (EII; Kinoshita et al. 1981), and endotype 6-aminohexanoate-oligomer hydrolase (EIII; Negoro et al. 1992; Kakudo et al. 1993, 1995), were responsible for the degradation of 6-aminohexanoate oligomers. The EI was a homodimer enzyme with subunits of M_r 52 kDa which was active only toward the cyclic dimer but not toward more than 100 kinds of natural amide bonds tested (Kinoshita et al. 1977). The EII was also a homodimer enzyme with subunits of M_r 42 kDa and was active on 6-aminohexanoate oligomers ranging from dimer to hexamer, but not on icosamer and hectamer. In addition, no activity was detected when the enzyme was tested with more than 100 kinds of possible amide bonds of natural compounds (Kinoshita et al. 1981). Furthermore, it was shown that the active site in the EII of KI72 involves a serine residue (Ser112; Negoro et al. 1989). The EIII enzyme was either a homodimer or a trimer with subunits of M_r 37 kDa. 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 so far tested (Negoro et al. 1992; Kakudo et al. 1993). The characteristics of the purified enzymes stated above are listed in Table 1. From the gathered experimental results, it was concluded that the degradation of various nylon oligomers followed the pathway shown in Fig. 1. The observed nylon oligomer degradative activities appeared to be high enough to make a biotechnological process possible.

Plasmid-dependence of nylon oligomer-degrading enzymes

Electron microscopy and restriction endonuclease analyses revealed that *Flavobacterium* sp. KI72 possessed three kinds of plasmids, pOAD1 [39 kilo-base-pairs (kb)], pOAD2 (44 kb) and pOAD3 (56 kb). Curing readily eliminated pOAD2 with the loss of the EI and EII enzyme activities. However, pOAD1 was either conserved intact (KI722), or only partially deleted (KI723), even after curing. Transformation of *Flavobacterium* sp. KI723 with the plasmid DNA of the wild strain showed that KI723 readily accepted the pOAD2 and simultaneously recovered its EI and EII enzyme activities (Fig. 2). These results demonstrated that the EI and EII enzymes were encoded on plasmid pOAD2 (Negoro et al. 1980). It was also shown that the EIII enzyme activities were detected in the wild type KI72 and transformant KI723T1, but not in the cured strain KI723 (Negoro et al. 1992).

Nucleotide sequence of pOAD2

The plasmid pOAD2 comprised 45,519 bp with a G + C content of 66.6% (GenBank/EMBL/DDBJ Accession No. D26094; Kato et al. 1995). Three nylon oligomer

Table 1 Characteristics of nylon oligomer-degrading enzymes from *Flavobacterium* sp. KI72. Specific activities are the values at a substrate concentration of 1 mM and are expressed as mol NH_2 liberated min^{-1} mg^{-1} purified protein (U mg^{-1}). *NT* Not tested

Characteristics	Enzymes			
	EI	EII	EIII	EII′
M_r of subunits	52 kDa	42 kDa	37 kDa	42 kDa
Number of amino acids	493	392	355	392
Optimum pH	7.4	9.0	7.0	NT
Optimum temperature	34 °C	40 °C	42 °C	NT
Gene	*nylA*	*nylB*	*nylC*	*nylB′*
Specific activity toward 6-aminohexanoate-				
linear-dimer	$<1.0 \times 10^{-3}$	0.94	4.4×10^{-4}	4.7×10^{-3}
linear-trimer	$<1.0 \times 10^{-3}$	0.75	0.11	NT
linear-tetramer	$<1.0 \times 10^{-3}$	0.57	0.42	NT
linear-pentamer	$<1.0 \times 10^{-3}$	0.24	0.47	NT
cyclic-dimer	2.8	$<1.0 \times 10^{-4}$	$<1.0 \times 10^{-4}$	NT
cyclic-tetramer	$<1.0 \times 10^{-3}$	$<1.0 \times 10^{-4}$	0.36	NT
ε-caprolactam	$<1.0 \times 10^{-3}$	$<1.0 \times 10^{-4}$	$<1.0 \times 10^{-4}$	NT

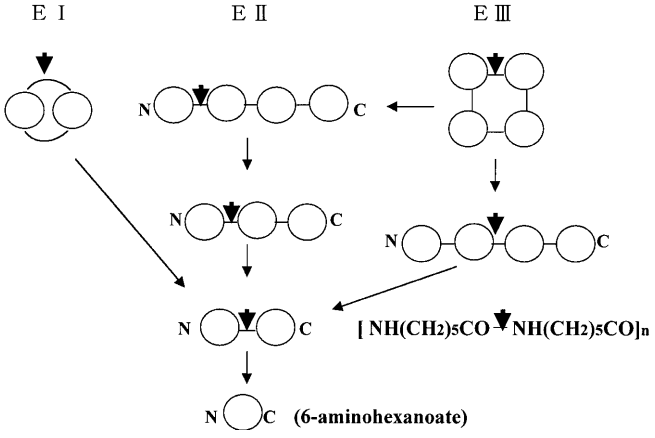

Fig. 1 Mode of degradation of nylon oligomers by 6-aminohexanoate-cyclic-dimer hydrolase (*EI*), 6-aminohexanoate-dimer hydrolase (*EII*) and endotype 6-aminohexanoate-oligomer hydrolase (*EIII*)

degradation genes, *nylA* (EI gene), *nylB* (EII gene), and *nylC* (EIII gene), were located on different parts of the plasmid (Fig. 3). In addition, the following DNA regions were found.

Repeated sequences

Southern hybridization experiments showed that at least two repeated sequence (RS-I and RS-II) were located on pOAD2 (Negoro et al. 1983). Sequence analysis revealed the organization of the repeated sequence was as follows:

1. RS-I. The sequences of the five RS-I regions identified on the plasmid were identical over 880 bp, except that the 420-bp region at the 3′ terminus of the 880-bp region was duplicated in RS-I_B (Kato et al. 1994). Two (RS-I_D and RS-I_E) had a reversed orientation on the map of pOAD2 (Fig. 3). In the 880-bp region, a 765-bp open reading frame (ORF) was observed to have 78-bp internal inverted sequences. A homology search of the DNA database 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 suggested that these elements were distributed widely among microorganisms, whether they be gram-negative or gram-positive bacteria.
2. RS-II. Two RS-II regions were found on pOAD2: RS-II_A contained the *nylB* gene, while RS-II_B contained the analogous *nylB′* gene. The *nylB* and *nylB′* had the same length ORF (1,176-bp), the same Shine–Dalgarno sequence, and the same position of initiation and termination codons. Of the 392 amino acid residues encoded by the ORF, only 46 residues were different. This hypothetical protein coded by the *nylB′* was named EII′. This result indicated that the *nylB* and *nylB′* genes evolved from the same ancestor gene. The EII′ had a very low catalytic activity (0.5% of EII activity) toward 6-aminohexanoate-dimer (Okada et al. 1983). These results suggested that an ancestral *nylB* gene on pOAD2 was duplicated and one copy mutated to increase the catalytic activity 200-fold.

Deduced gene product showing significant homology to extant protein

Identification of the promoter regions and Northern hybridization experiments demonstrated that *nylB* and *nylC* seemed to constitute an operon with another unknown gene (Okazaki et al. 1988; Zhou et al. 1990). After a homology search, we found that in between the promoter region and the *nylC* gene, there was a DNA region homologous to the oligopeptide uptake (*opp*) gene clusters (Hiles et al. 1987; Alloing et al. 1990; Perego et al. 1991; Rudner et al. 1991). Oligopeptide permease is considered to constitute a complex of ATPase, inner membrane protein, and binding protein (Hiles et al. 1987; Perego et al. 1991). OppF and OppD, having the ATPase activity, are localized in the cytoplasm, while OppB and OppC are membrane proteins. OppA is assumed to be the initial binding site for substrates. Since nylon oligomers and oligopeptides are

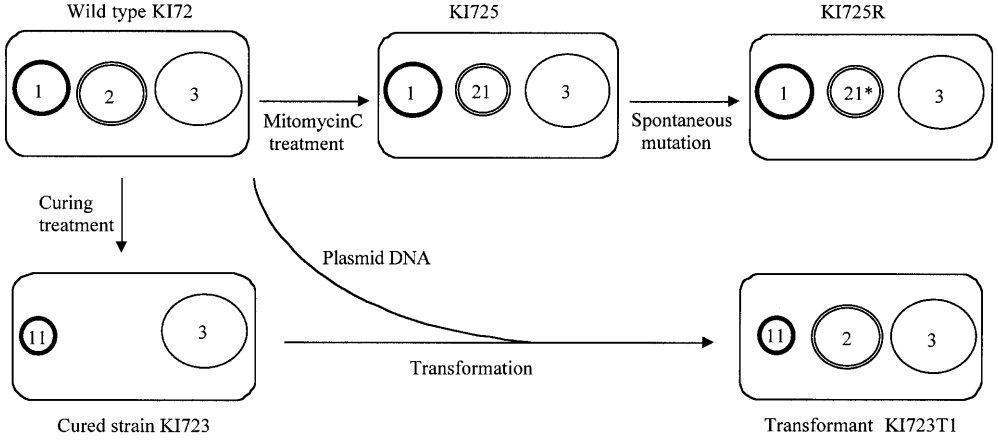

Fig. 2 The genetic steps for the formation of cured and transformant strains. *1* pOAD1, *11* pOAD11 (a deletion plasmid from pOAD1), *2* pOAD2, *21* pOAD21 (a deletion plasmid from pOAD2; see Fig. 3), *21** pOAD21 having Nom^+ spontaneous mutation (see text), *3* pOAD3

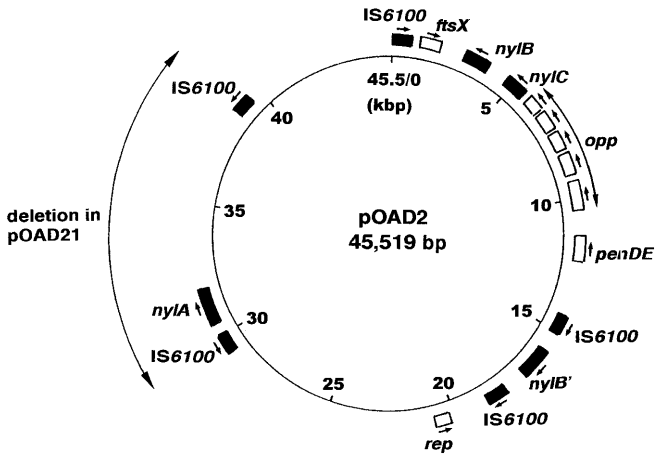

Fig. 3 Deduced functional map of pOAD2. *Heavy boxes* represent the regions encoding the identified gene products, i.e., four nylon oligomer degradation genes [*nylA* 30,359–31,840 (position of initiation codon – position of termination codon), *nylB* 3,989–2,811, *nylB′*, 15,869–17,047, *nylC* 5,669–4,602] and five IS*6100*-transposase genes [positions: 41–805 (RS-I_A), 14,366–15,130 (RS-I_B), 17,910–18,674 (RS-I_C), 29,973–29,209 (RS-I_D), 39,515–38,751 (RS-I_E)]. In RS-I_B, the 420-bp region at the 3′ terminus (position: 15,185–15,604) was a repeated portion of the IS*6100* element. *Open boxes* represent the DNA regions which have significant homology with the sequences of the *oppA-F*, *ftsX*, *penDE*, and *rep* genes. Plasmid pOAD21 was obtained from pOAD2 by homologous recombination between the directly repeated IS*6100* sequences. kbp Kilo base pairs

analogous compounds in which monomeric units having an amino group and a carboxy group are combined by amide bonds, the gene clusters on pOAD2 might be involved in the transport of nylon oligomers.

In addition, DNA regions homologous to the *ftsX* (filamentation temperature sensitivity; Gill et al. 1986), *penDE* (isopenicillin *N*-acyltransferase; Tobin et al. 1990; Alvarez et al. 1993), and *rep* (replication) genes of various plasmids (Mori et al. 1986; Thomas and Smith 1986; Tabata et al. 1989; Davis et al. 1992) were found on pOAD2. Figure 3 illustrates the functional map of pOAD2.

Amino acid alterations increasing the catalytic activity of nylon oligomer-degrading enzymes

Though the EII and EII′ had the same number of amino acid residues and were similar in their amino acid sequence, their catalytic activity was quite different. To identify essential amino acid alterations which should be involved in the evolution of the EII enzyme, various hybrid genes between the *nylB* and *nylB′* were constructed (Negoro et al. 1984; Fujiyama et al. 1991; Kato et al. 1991). These results demonstrated that just two amino acid replacements, i.e., Gly181 to Asp (EII type) and His266 to Asn (EII type), in the EII′ protein sufficed to increase the activity of EII′ to the level of the parent EII enzyme. Furthermore, it was also shown that the remaining 44 amino acid substitutions played no part in enzyme activity, although these 44 alterations included many non-synonymous substitutions (Kato et al. 1991). In addition, replacement of Asp181 with Asn, Glu, His, or Lys by site-directed mutagenesis suggested that alterations at this position affected the binding of the substrate to the enzyme (Hatanaka et al. 1991). Thus, very few amino acid alterations seemed to be required for an enzyme to evolve, such as the evolution of EII from its antecedent. Microorganisms may possess a cryptic gene such as the EII′ gene; and by increasing the activity of an enzyme degrading a xenobiotic compound, the microorganisms may be able to adapt toward the utilization of that compound.

Biodegradation of nylon oligomers in *Pseudomonas*

Pseudomonas sp. NK87 harbored six kinds of plasmid, namely: pNAD1 (20 kb), pNAD2 (23 kb), pNAD3 (51 kb), pNAD4 (57 kb), pNAD5 (76 kb), and pNAD6 (80 kb). Cloning and hybridization experiments revealed that the *nylA* and *nylB* genes of NK87 were located on pNAD2 and pNAD6, respectively (Kanagawa et al. 1989). The EI enzyme from NK87 (P-EI) was composed of 493 amino acid residues, which was identical to the length of the EI from *Flavobacterium* sp. KI72 (F-EI). The amino acid sequence of the P-EI enzyme was almost identical to the sequence of the F-EI (99% homology; Tsuchiya et al. 1989). However, the EII had more divergent sequences. The homology between the EII sequence of KI72 (F-EII) and that of NK87 (P-EII) was only 35% with respect to the amino acid sequence (Kanagawa et al. 1993). The P-EII was composed of 396 amino acids and was inhibited by a serine protease inhibitor, diisopropylfluorophosphate, as was the case with F-EII. The P-EII enzyme had higher affinity toward 6-aminohexanoate-dimer (K_m for P-EII, 0.6 mM; K_m for F-EII, 15 mM), while the k_{cat} value of the EII enzyme (9.2 s^{-1}) was approximately half that of the value of the F-EII enzyme (19 s^{-1}; Kanagawa et al. 1993). The EIII enzyme has not been detected in strain NK87.

Sequences similar to IS*6100* were also found in the plasmid pNAD2, which contains the *nylA* gene in *Pseudomonas* sp. NK87, but not in the other five plasmids, nor in the chromosome of NK87. The absence of sequence diversity in the IS elements indicated 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, suggested that the IS sequence provided some growth advantage for the host strains to select the *nylA* gene.

Experimental evolution of nylon oligomer-degrading enzymes

If we could directly evolve a new metabolic ability under laboratory conditions, it would be interesting from the standpoint of enzyme evolution; and it would also pro-

vide a good system for studying the adaptation of microorganisms toward xenobiotic compounds. Here, we describe the artificial expansion of the metabolic ability of *Flavobacterium* sp. and *Pseudomonas aeruginosa*.

A derivative of KI72 was used, namely KI725, containing pOAD21 (a deletion plasmid of pOAD2). The 9-kb deletion containing the entire *nylA* region was caused by recombination between the two IS*6100* elements (Negoro and Okada 1982; Kato et al. 1994; Fig. 3). Since strain KI725 does not grow on unfractionated nylon oligomer (Nom), which includes 6-aminohexanoate-cyclic oligomers as major components, we tested whether this strain could restore the ability to metabolize Nom. When KI725 cells were spread on Nom-minimal medium, colonies appeared spontaneously (Nom^+ phenotype, KI725R strains) at a frequency of 10^{-7} per cell after 14 days of incubation. Cell extracts from Nom^- strains had low levels of enzyme activity with Nom as a substrate (ca. 5% of the activity of KI72). However, this activity was two- to six-fold higher in the Nom^+ revertants. Therefore, it is conceivable that the elevated levels of the enzyme activities responsible for Nom degradation made the cells Nom^+ (Fig. 2).

To see whether nylon oligomer degradation occurs in bacteria not related to *Flavobacterium* species, we tested *Pseudomonas aeruginosa* PAO, a standard *Pseudomonas* strain (Holloway and Morgan 1986). 6-Aminohexanoate was poorly utilized, whereas neither its linear dimer nor cyclic dimer was utilized by the wild type PAO1. A mutant PAO5501 which grows fast on 6-aminohexanoate as a sole source of carbon and nitrogen was initially isolated from PAO1; and then a mutant which grows on both the cyclic dimer and the linear dimer (designated PAO5502) was obtained from PAO5501 (Prijambada et al. 1995). The enzyme assay demonstrated that the ability of PAO5502 to utilize the cyclic dimer was due to the existence of a newly evolved enzymatic system responsible for degrading the cyclic dimer.

Molecular basis for adaptation toward a xenobiotic compound

Several molecular mechanisms are possible for microorganisms to adapt toward non-physiological substrates: (1) alteration of substrate specificity of an enzyme (Clarke and Slater 1986), (2) activation of a cryptic gene by mutation (Hall and Clarke 1977), (3) alteration of regulator specificity (Ramos et al. 1986), and (4) alteration of uptake specificity. Two hypotheses for the birth of nylon oligomer degradation genes were proposed. Namely:

1. The EII enzyme is specified by an alternative ORF of the preexisting coding sequence that originally specified a 472-residue-long arginine-rich protein, and a frame shift mutation in the ancestral gene creates the new gene (Ohno 1984).
2. There is some special mechanism which protects a nonstop frame (NSF), namely a long stretch of a sequence without chain-terminating base triplets, from mutations that generate the stop codons on the antisense strand; and such a mechanism enables the NSF to evolve into new functional genes (Yomo et al. 1992).

Although the molecular basis of nylon oligomer metabolism in strain PAO5502 is still unknown, it could be that environmental stress favors this adaptation. DNA polymerase errors are known to give rise to some "adaptive" mutations, converting from a Lac^- phenotype to Lac^+ phenotype in *Escherichia coli* (Foster and Trimarch 1994; Rosenberg et al. 1994). Furthermore, recombination appears to be involved in the phenomenon of "adaptive" mutation (Harris et al. 1994). A 10,000-fold increase in Mu element excision was observed due to starvation (Lenski and Mittler 1993). Since the nylon oligomer has no detectable toxicity toward microorganisms, the wild type cells could be maintained in the starved condition for a long period. After the cells accumulated the required genetic alteration to make a cryptic region active, cells grew in the nylon oligomer medium. Our experimental results indicated that the metabolic range of a bacterium could be expanded to include the nylon oligomer degradation.

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