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A New Nylon Oligomer Degradation Gene (*nylC*) on Plasmid pOAD2 from a *Flavobacterium* sp.

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***Flavobacterium* sp. strain KI725 harbors plasmid pOAD21, a derivative of nylon oligomer-degradative plasmid pOAD2, in which all of *nylA* (the gene for 6-aminohexanoate cyclic dimer hydrolase [EI]) was deleted but *nylB* (the gene for 6-aminohexanoate dimer hydrolase [EII]) was retained. KI725 showed no growth on unfractionated nylon oligomers (Nom1) obtained from a nylon factory as a sole carbon and nitrogen source (Nom1 minimum plate). Extracts of KI725 cells possessed hydrolytic activity for Nom1 (approximately 5% of the activity of KI72), but pOAD2-cured strains (KI722 and KI723) showed no activity. KI725R strains which grew on the Nom1 minimum plate were spontaneously isolated from KI725 at a frequency of 10^{-7} per cell. Activity toward Nom1 was enhanced in KI725R strains (10 to 30% of the activity of KI72). This new Nom1 degrading enzyme (EIII, the *nylC* gene product) hydrolyzed not only Nom1 but also the *N*-carbobenzoxy-6-aminohexanoate trimer, a substrate which was not hydrolyzed by either EI or EII. Cloning and sequence analysis showed that the *nylC* gene is located close to *nylB* on pOAD21 and is a 1,065-bp open reading frame corresponding to 355 amino acid residues. The nucleotide sequence of the *nylC* gene and the deduced amino acid sequence of EIII had no detectable homology with the sequences of *nylA* (EI) and *nylB* (EII).**

Enzymes responsible for the degradation of man-made compounds are interesting from an evolutionary point of view. We have isolated bacterial strains, *Flavobacterium* sp. strain KI72 (5) and *Pseudomonas* sp. strain NK87 (4), that grow on the 6-aminohexanoate cyclic dimer (AC2), a by-product of nylon factories. Two enzymes, AC2 hydrolase (EI) (6) and 6-aminohexanoate dimer (AL2) hydrolase (EII) (7), have been found to be responsible for degradation of the cyclic dimer. The EI enzyme is specifically active on AC2 (6); the EII enzyme is active on 6-aminohexanoate linear oligomers (degree of polymerization, 2–20) but not on the *N*-carbobenzoxy derivatives of the linear oligomers (7, 10). The EI-encoding gene (F-*nylA*) and EII-encoding gene (F-*nylB*) of *Flavobacterium* sp. strain KI72 are located on plasmid pOAD2 (44 kb), one of the three plasmids harbored in strain KI72 (13, 14, 15). In *Pseudomonas* sp. strain NK87, the EI gene (P-*nylA*) and EII-encoding gene (P-*nylB*) are located on different plasmids, pNAD2 and pNAD6, respectively (4, 19).

We have reported that strain KI725, obtained by mitomycin C treatment of KI72, grows on AL2 (Ald$^+$) but not on AC2 (Acd$^-$) (12). KI725 contained pOAD21 (35 kb), a deletion plasmid derived from pOAD2, and showed no growth on unfractionated nylon oligomers (Nom1) obtained from a nylon factory. However, derivative strains of KI725 which grew on a Nom1 minimum plate appeared spontaneously (KI725R strains). These results suggest that a new type of nylon oligomer-degrading enzyme is present in strain KI725 and/or KI725R.

Here we report the cloning, genetic organization, and nucleotide sequence of a new nylon oligomer degradation gene, *nylC*, found in pOAD21.

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MATERIALS AND METHODS

Enzymes and chemicals. Restriction endonucleases (*Bgl*II, *Eco*RI, *Hin*dIII, *Pst*I, *Sal*I, and *Sma*I) and T4 DNA ligase were obtained from Toyobo Co. Ltd. AL2 and the *N*-carbobenzoxy-6-aminohexanoate trimer (Z-AL3) were chemically synthesized by a previously described method (5, 7). Two different batches of unfractionated nylon oligomers (Nom1 and Nom2) were generous gifts of Toyobo Co. (Osaka, Japan). Nom1 includes AC2 as a main component and also contains a small amount of the 6-aminohexanoate cyclic oligomers (higher degree of polymerization). Nom2 is a nylon oligomer mixture rich in higher-degree cyclic oligomers. AC2 was purified from Nom1 by crystallization with hot water (6). Other chemicals were guaranteed grade reagents from Nacalai Tesque Co. Ltd.

Media. Luria-Bertani (LB) broth (17) was used as complete medium for all strains. The minimum agar media used for *Flavobacterium* and *Pseudomonas* strains contained 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 in common and 2 g of AC2 (for AC2 minimum plates), 2 g of AL2 (for AL2 minimum plates), 5 g of Nom1 (for Nom1 minimum plates), or 5 g of Nom2 (for Nom2 minimum plates) per liter as the sole carbon and nitrogen source. For selection of nylon oligomer-degradative activities on plates, LB agar media containing the following nylon oligomers were autoclaved and then cooled rapidly in a refrigerator to obtain fine, insoluble particles of the oligomers: 15 g of AC2 (LB-AC2 plate) per liter, 5 g of Z-AL3 (LB–Z-AL3 plate) per liter, or 15 g of Nom2 (LB-Nom2 plate) per liter. When necessary, 50 μg of ampicillin per liter was added.

Microorganisms and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. *Flavobacterium* strain KI725, which grew on AL2 minimum plates but not on Nom1 minimum plates, was obtained from KI72 by mitomycin C (0.5 μg/ml) treatment (12). KI7250 is a streptomycin-resistant mutant of KI725 isolated on LB me-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristic(s)[a]	Reference
Flavobacterium sp. strains		
KI72 (wild type)	Acd^+ Ald^+ Ahx^+ Nom^+ (pOAD1, pOAD2, pOAD3)	5
KI722	Acd^- Ald^- Ahx^+ Nom^- (pOAD1, pOAD3)	13
KI723	Acd^- Ald^- Ahx^{++} Nom^- (pOAD11, pOAD3)	13
KI725	Acd^- Ald^+ Ahx^+ Nom^- (pOAD1, pOAD21, pOAD3)	12
KI7250	Acd^- Ald^+ Ahx^+ Nom^- Str^r (pOAD1, pOAD21, pOAD3)	This study
KI725R1	Acd^- Ald^+ Ahx^+ Nom^+ (pOAD1, pOAD21*, pOAD3)	This study
KI7250R1	Acd^- Ald^+ Ahx^+ Nom^+ Str^r (pOAD21′, pOAD3)	This study
KI7250R2	Acd^- Ald^+ Ahx^+ Nom^+ Str^r (pOAD1, pOAD21′, pOAD3)	This study
KI723T1	Acd^+ Ald^+ Ahx^{++} Nom^+ (pOAD11, pOAD2, pOAD3)	13
KI723T11	Acd^- Ald^+ Ahx^{++} Nom^- (pOAD11, pOAD21, pOAD3)	This study
KI723T12	Acd^- Ald^+ Ahx^{++} Nom^+ (pOAD11, pOAD21*, pOAD3)	This study
Pseudomonas sp. strain NK87	Acd^+ Ald^+ Ahx^+ Nom^- (pNAD1, pNAD2, pNAD3, pNAD4, pNAD5, pNAD6)	4
E. coli strains		
$C600r_K^-m_K^-$	*thr-1 leuB6 thi-1 supE44 lacY1 tonA21 hsdM hsdR*	1
JM103	*Δ(lac-pro) thi strA supE endA sbcB15 hsdR4* (F′ *traD36 proA⁺B⁺ lacI^q lacZΔM15*)	8
Plasmids		
pBR322	Amp^r Tet^r	3
pUC18	Amp^r	22
pUC19	Amp^r	22
pOAD1	Cryptic plasmid (40 kb)	13
pOAD2	Nylon oligomer-degradative plasmid (44 kb) *nylA⁺ nylB⁺ nylC⁺*	13
pOAD3	Cryptic plasmid (56 kb)	13
pOAD11	Deletion plasmid from pOAD1 (36 kb)	13
pOAD21	Deletion plasmid from pOAD2 (35 kb) *ΔnylA nylB⁺ nylC⁺*	12
pOAD21*	35-kb plasmid in Nom^+ strains (KI725R1, KI723T12) *ΔnylA nylB⁺ nylC⁺*	This study
pOAD21′	35-kb plasmid in Nom^+ strains (KI7250R1, KI7250R2) *ΔnylA nylB⁺ nylC⁺*	This study

[a] Acd, AC2; Ald, AL2; Ahx, 6-aminohexanoate; Nom, Nom1; Str, streptomycin; Amp, ampicillin; Tet, tetracycline. Hypergrowth is shown as ++. It was confirmed that pOAD21* transforms KI723 to Nom^+, but it was not confirmed that pOAD21′ does. A cryptic plasmid, pOAD1, is spontaneously lost in KI7250R1.

dium containing 100 μg of streptomycin per ml. KI725 and KI7250 cells were spread on Nom1 minimum plates (3×10^8 cells per plate) and incubated at 30°C for 2 weeks. Colonies which appeared (Nom^+ phenotype) were purified on Nom1 minimum plates. Two Nom^+ strains (KI725R1 and KI725R2) from KI725 and two Nom^+ strains (KI7250R1 and KI7250R2) from KI7250 were used.

Preparation of a crude enzyme solution. Cells grown on 100 ml of LB medium were washed with 20 mM potassium phosphate buffer (pH 7.3) containing 10% glycerol (buffer A) and suspended in 5 ml of buffer A. The cell suspension was sonicated at 20 kHz for 8 min (Kaijo-Denki 4280 ultrasonicator) and centrifuged at $10,000 \times g$ for 5 min. The supernatant obtained was used as a crude enzyme solution.

Assay. For qualitative detection of enzyme activities, enzyme preparations were incubated with 10 mM AC2 (EI assay), 10 mM AL2 (EII assay), or 2.5 mg of Nom1, Nom2, or Z-AL3 per ml at 30°C in buffer A and the reaction products (2 μl) were spotted on thin-layer silica gel sheets ($60F_{254}$; Merck Co. Ltd.), developed with *n*-butanol–acetic acid–water (4:1:2), and detected with ninhydrin (0.2% in *n*-butanol) (6). To assay activity quantitatively, enzyme reactions were done at 30°C by using 10 mM AC2 (EI assay), 1.5 mM AL2 (EII assay), or 2.5 mg of Nom1 per ml in buffer A as the substrate, and the increase of amino groups was measured by the trinitrobenzene sulfonate method (6). Proteins were measured by A_{280}, assuming that 1 absorbance unit corresponds to 0.5 mg/ml.

DNA manipulation. *Escherichia coli* plasmid DNA was prepared by the alkaline extraction method (2) followed by purification by CsCl-ethidium bromide density gradient centrifugation (17). Plasmids of *Flavobacterium* strains were similarly prepared but with some modifications (13). Restriction endonuclease digestions, ligation, and transformation were done by conventional methods (17).

Electrophoresis. Agarose gel electrophoreses were done by the method of Sambrook et al. (17), with 0.55 and 1% gels 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 (17). DNA fragments were recovered from agarose gels by the method of Vogelstein and Gillespie (20), by using a Geneclean kit (Bio101, Inc.).

Detection of an *E. coli* clone possessing the *nylC* gene. For cloning of the *nylC* gene, pOAD21* of strain KI725R1, fractionated by agarose gel electrophoresis, was digested with *Hin*dIII and the restriction fragments obtained were ligated with pBR322. *E. coli* $C600r_K^-m_K^-$ was transformed with the ligated DNA by the $CaCl_2$ method (17). Transformants possessing EIII activity were selected as a clone producing a halo on an LB–Z-AL3 plate containing 50 μg of ampicillin per ml.

Nucleotide sequence analysis. The nucleotide sequence of the 1.3-kb *Hin*dIII-*Bam*HI fragment of pULC3 containing the *nylC* gene was determined by the dideoxynucleotide chain termination method (18). Subfragments were prepared from the 1.3-kb fragment by using nuclease BAL 31 (17) and inserted into M13mp18 and M13mp19 (22). The nucleotide sequences of the subfragments which start by 100- to 200-bp intervals were determined by using sequencing kits (Toyobo

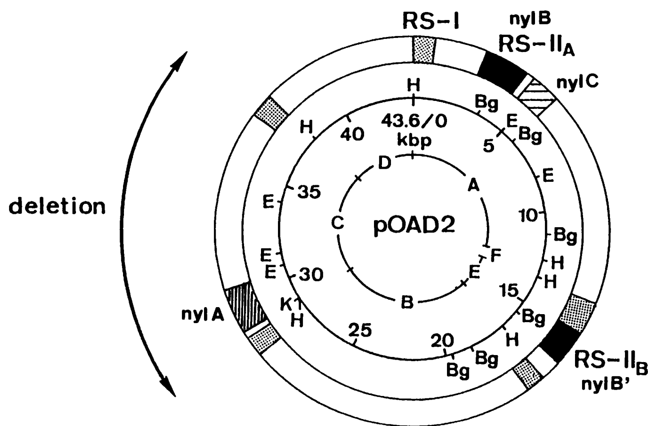

FIG. 1. Structural and functional map of plasmid pOAD2. The nylon oligomer degradation genes were at the following loci: *nylA*, 29.1- to 30.6-kb region on the pOAD2 map; *nylB*, 2.7- to 4.1-kb region; *nylB'*, 14.7- to 16.1-kb region; *nylC*, 4.2- to 5.3-kb region. RS-I and RS-II are repeated sequences identified by Southern hybridization experiments. RS-I appeared five times on pOAD2 (RS-I_A, 0 to 0.8 kb; RS-I_B, 13.5 to 14.7 kb; RS-I_C, 16.8 to 17.4 kb; RS-I_D, 28.2 to 28.8 kb; RS-I_E, 37.1 to 37.9 kb). The *nylB* and *nylB'* genes are included in the RS-II_A and RS-II_B regions, respectively. Bg, E, H, and K represent *Bgl*II, *Eco*RI, *Hin*dIII, and *Kpn*I restriction sites, respectively. pOAD21 was obtained from pOAD2 by homologous recombination between direct-repeat sequences RS-I_D and RS-I_E. The arrow indicates the region deleted in pOAD2.

Co.). Sequences were identified for both strands of the 1.3-kb region.

Nucleotide sequence accession number. The sequence reported here has been deposited in the GenBank data base (accession no. D10686).

RESULTS

Evidence for the presence of a new nylon oligomer degradation gene (*nylC*) in a *Flavobacterium* sp. *Flavobacterium* sp. strain KI725 harbors a deletion plasmid (pOAD21 [35 kb]) derived from pOAD2 (12). The 9-kb deleted region includes the entire *nylA* region, and the ends of the deletion were in repeated sequences RS-I_D and RS-I_E (Fig. 1) (14); therefore, KI725 grows on AL2 but not on AC2. This strain cannot grow on an unfractionated nylon oligomer (Noml). However, when KI725 cells were spread on a Noml minimum plate, colonies appeared spontaneously (Nom$^+$ phenotype, KI725R strains) after 14 days of incubation at a frequency of 10^{-7} per cell (Fig. 2A). These results suggest that a new gene responsible for metabolism of a different type of nylon oligomer is included in KI725R strains. Two Nom$^+$ strains (KI725R1 and KI725R2) obtained from KI725 and two Nom$^+$ strains (KI7250R1 and KI7250R2) from KI7250 (streptomycin-resistant mutant of KI725) were selected for further study.

An Ald$^+$ transformant (KI723T11) obtained from KI723 by the $CaCl_2$ method (13) by using a plasmid mixture of KI725 showed no growth on a Noml minimum plate. However, plasmids from KI725R1 could transform KI723 to Nom$^+$, and in one of the Nom$^+$ transformants, KI723T12, a 35-kb plasmid was detected in addition to two plasmids (pOAD11 and pOAD3) derived from the recipient (Table 1). These results suggest that mutations were on pOAD21. To distinguish the 35-kb plasmid of KI725R1 from pOAD21 of

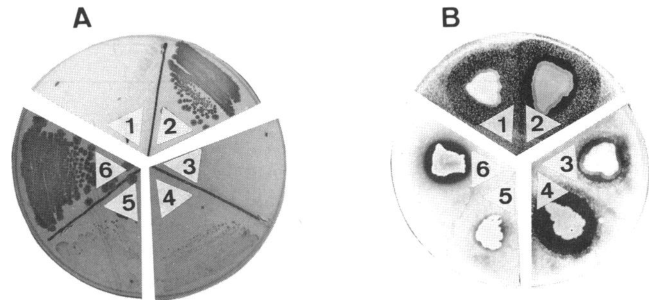

FIG. 2. Growth of *Flavobacterium* and *Pseudomonas* strains on selection plates. (A) *Flavobacterium* sp. strain KI72 and its derivative strains were inoculated on an AC2 minimum plate (sections 1 and 2) and a Noml minimum plate (sections 3, 4, 5, and 6) and incubated for 7 days at 30°C. Sections: 1, KI725R1; 2, KI72; 3, KI725; 4, KI725R1; 5, KI725R2; 6, KI72. (B) *Pseudomonas* sp. strain NK87 (sections 1, 3, and 5) and *Flavobacterium* sp. strain KI72 (sections 2, 4, and 6) were inoculated on LB-Nom2 (sections 1 and 2), LB-AC2 (sections 3 and 4), and LB-Z-AL3 (sections 5 and 6) plates and incubated for 7 days at 30°C.

KI725, the former plasmid was tentatively named pOAD21*. Since the 35-kb plasmid fractions recovered from the KI725, KI7250, KI725R1, KI7250R1, and KI7250R2 strains exhibited identical patterns for *Hin*dIII and *Eco*RI digestions, some minor genetic alterations, such as point mutations, seem to occur on these plasmids.

We prepared cell extracts from two pOAD2-harboring strains (KI72 and KI723T1), two pOAD2-cured strains (KI722 and KI723), two Acd$^-$ Ald$^+$ Nom$^-$ strains (KI725 and KI7250), and three Nom$^+$ mutant strains (KI725R1, KI7250R1, and KI7250R2) and examined their enzyme activities (Fig. 3). Hydrolysis of AC2 was observed only for the two pOAD2-harboring strains and not for the other seven Acd$^-$ strains (data not shown). When Noml was used as the substrate, high activities were detected in pOAD2-harboring strains (KI72, 0.02 μmol/min/mg [U/mg]; KI723T1, 0.017 U/mg)] and no activity was detected in pOAD2-cured strains

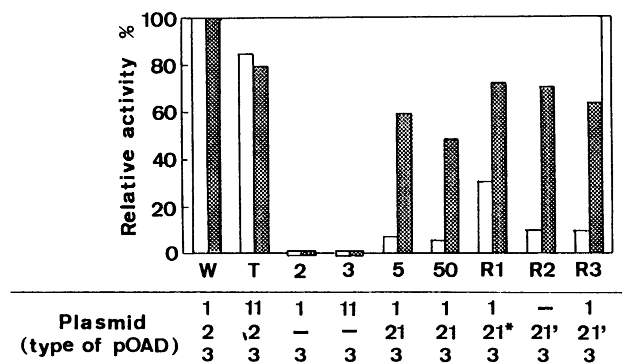

FIG. 3. Activities of nylon oligomer-degrading enzymes in *Flavobacterium* sp. strain KI72 and derivative strains. All strains were grown in LB medium, and cell extracts were obtained by sonication followed by centrifugation. Enzyme activities were measured by using 1.5 mM AL2 (▥) (for EII assay) and 2.5 mg of Noml per ml (□). Enzyme activity is expressed as a percentage of the activity of KI72 toward each substrate (activity toward AL2, 0.004 μmol/min/mg [U/mg]; activity toward Noml, 0.02 U/mg). W, KI72; T, KI723T1; 2, KI722; 3, KI723; 5, KI725; 50, KI7250; R1, KI725R1; R2, KI7250R1; R3, KI7250R2.

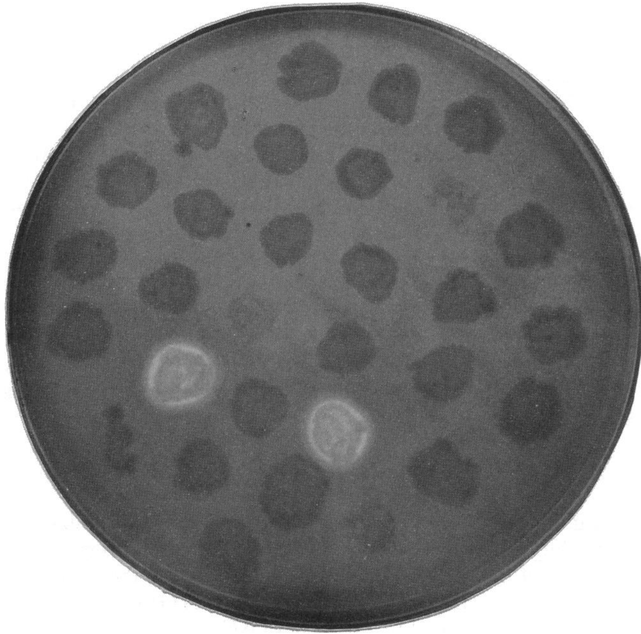

FIG. 4. Detection of EIII$^+$ transformants on an LB–Z-AL3 plate. Plasmid pOAD21* from strain KI725R1 was digested with *Hin*dIII. The fragments were ligated with pBR322, which had been digested with *Hin*dIII and treated with alkaline phosphatase. The ligation mixture was used for transformation of *E. coli* C600$r_K^-m_K^-$. Ampicillin-resistant transformants were incubated on LB–Z-AL3 medium containing 50 μg of ampicillin per ml and incubated for 7 days. EIII$^+$ transformants were selected as a clone which produced a clear zone around the colony.

(<0.001 U/mg). However, approximately 5% of the activity of KI72 was detected in strains KI725 and KI7250, and the activity was enhanced two- to sixfold in strains KI725R1, KI7250R1, and KI7250R2 (10 to 30% of the activity of KI72). These results suggest that KI725 and/or its derivative strains possess at least one new type of nylon oligomer-degrading enzyme (EIII) which is active toward Nom1.

Cloning of the *nylC* gene in *E. coli*. Since preliminary experiments with a partially purified EIII enzyme from strain KI725R1 suggests that this enzyme is active toward Z-AL3, we attempted to clone the EIII gene (*nylC*) in *E. coli* by selecting clones which have hydrolytic activity toward Z-AL3. Plasmid pOAD21* of strain KI725R1, fractionated by agarose gel electrophoresis, was digested with *Hin*dIII, and the *Hin*dIII fragments (A, B, D, E, and F [Fig. 1]) were ligated with pBR322, which had been digested with *Hin*dIII; this was followed by transformation of *E. coli* C600$r_K^-m_K^-$. Transformants harboring plasmid pKN1, which contains the *Hin*dIII A fragment as an insert, made a clear zone on an LB–Z-AL3 plate (Fig. 4). Crude extracts of *E. coli*(pKN1) gave a major AL1 spot from Z-AL3 on thin-layer chromatography. However, transformants containing *Hin*dIII-B, -D, -E, and -F had no hydrolytic ability toward Z-AL3. Since the EII enzyme has no activity toward Z-AL3 (7), hydrolysis of Z-AL3 is not dependent on the EII enzyme expressed in *E. coli*(pKN1). These results suggest that the *nylC* gene was cloned in the *Hin*dIII A fragment.

To localize the gene on the A fragment, we constructed deletion plasmids from pKN1 (Fig. 5). Hydrolysis of Z-AL3 was detected in pULC2, which contains the 1.4-kb *Bgl*II-*Pst*I fragment between the *Bam*HI and *Pst*I sites of pUC18, but not in a plasmid (pULC2R) containing the same *Bgl*II-*Pst*I fragment between the *Bam*HI and *Pst*I sites of pUC19, in which orientation of the fragment with respect to the *lac* promoter was reversed. This suggests that the *nylC* gene is expressed under control of the *lac* promoter in pUC19 and that the directions of the *nylB* and *nylC* genes are the same on pOAD21. The cell extracts of *E. coli* JM103 harboring the smallest plasmid, pULC3, produced a major AL2 spot and some minor spots, such as AL1, from Nom1, Nom2, and Z-AL3 after 16 h of incubation, but the extracts gave no reaction products from AC2 and AL2. In a control experi-

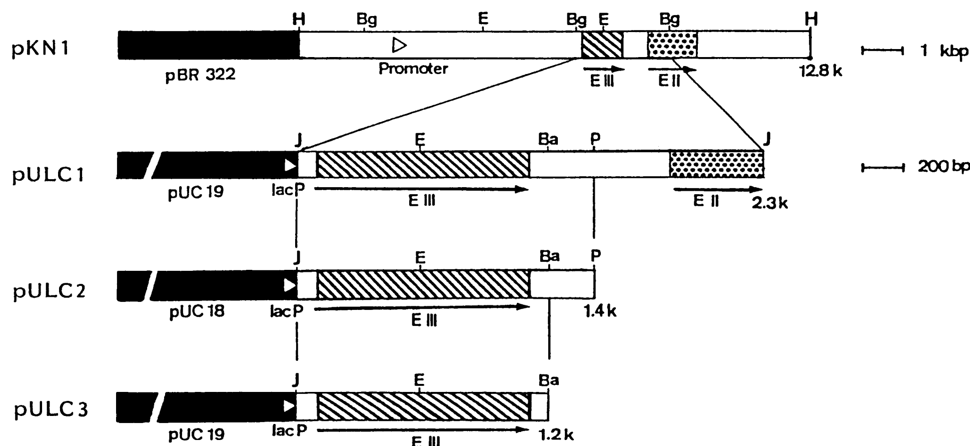

FIG. 5. Deletion plasmid analysis of the *nylC* gene locus. Plasmid pKN1 was composed of vector pBR322 and the 12.8-kb *Hin*dIII fragment of pOAD21*. The 2.3-kb *Bgl*II fragment of pKN1 was cloned into the unique *Bam*HI site of pUC19, where pULC1 was obtained. Plasmid pULC2 is composed of the 1.4-kb *Bgl*II-*Pst*I fragment that encodes the *nylC* gene and the 2.6-kb *Bam*HI-*Pst*I fragment of pUC18. Plasmid pULC3 was obtained by inserting the 1.2-kb *Bgl*II-*Bam*HI fragment of pULC2 into a *Bam*HI site of pUC19. Cell extracts of *E. coli* C600$r_K^-m_K^-$(pKN1) or JM103 harboring pULC1, pULC2, or pULC3 were incubated with 2.5 mg of Nom1, Nom2, or Z-AL3 per ml at 30°C for 16 h, and reaction products were analyzed by thin-layer chromatography. All *E. coli* clones possessed hydrolytic activity. B, Bg, E, H, and P indicate *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, and *Pst*I restriction sites, respectively. J indicates a junction obtained by connection of *Bgl*II and *Bam*HI cohesive ends. The triangle indicates the *lac* promoter region contained in the pUC vector (22). The location and direction of the *nylC* gene are shown by arrows.

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BglII 10        20        30        40        50        60        70        80        90
AGATCTCGTCGAGACCGCCGCGACCGACGAGTTCTTCCGGAACCCCCGGCATCCCTACTCGCAGACACTCTTGGAGGCCCTATGAATACG
                                                                                 MetAsnThr

                                                                                      180
ACACCGGTCCACGCACTCACCGACATCGACGGCGGGATCGCCGTCGATCCCGCACCCCGGCTGGCCGGCCCTCCGGTCTTCGGGGGTCCG
ThrProValHisAlaLeuThrAspIleAspGlyGlyIleAlaValAspProAlaProArgLeuAlaGlyProProValPheGlyGlyPro

                                                                        SmaI          270
GGCAACGACGCCTTCGATCTCGCGCCGGTCAGGAGCACGGGCCGCGAGATGCTGCGGTTCGACTTCCCCGGGGTCAGCATCGGCGCGGCG
GlyAsnAspAlaPheAspLeuAlaProValArgSerThrGlyArgGluMetLeuArgPheAspPheProGlyValSerIleGlyAlaAla

                                                                                      360
CACTACGAGGAGGGGCCCACCGGTGCGACCGTGATCCACATCCCCGCCGGCGCCCGCACCGCGGTGGACGCGCGGGGCGGGGCGGTGGGG
HisTyrGluGluGlyProThrGlyAlaThrValIleHisIleProAlaGlyAlaArgThrAlaValAspAlaArgGlyGlyAlaValGly

                                                                                      450
CTCTCCGGCGGCTACGACTTCAACCACGCCATCTGCCTGGCCGGCGGAGCCGGCTACGGGCTCGAGGCGGGCGCCGGGGTGAGCGACGCG
LeuSerGlyGlyTyrAspPheAsnHisAlaIleCysLeuAlaGlyGlyAlaGlyTyrGlyLeuGluAlaGlyAlaGlyValSerAspAla

                                     SacI                                             540
CTCCTGGAACGCCTCGAGCATCGCACCGGCTTCGCCGAGCTCCAGCTGGTGTCGTCGGCGGTCATCTACGACTTCTCGGCGCGCTCCACC
LeuLeuGluArgLeuGluHisArgThrGlyPheAlaGluLeuGlnLeuValSerSerAlaValIleTyrAspPheSerAlaArgSerThr

                                       EcoRI                                          630
GCGGTCTACCCCGACAAGGCGCTCGGCCGCGCGGCGCTCGAATTCGCCGTTCCCGGTGAGTTCCCGCAGGGGCGGGCGGGCGCGGGCATG
AlaValTyrProAspLysAlaLeuGlyArgAlaAlaLeuGluPheAlaValProGlyGluPheProGlnGlyArgAlaGlyAlaGlyMet

                                                                                      720
AGCGCGTCCGCGGGCAAGGTGGACTGGGACCGCACCGAGATCACCGGGCAGGGCGCGGCGTTCCGTCGTCTCGGCGACGTGCGCATCCTC
SerAlaSerAlaGlyLysValAspTrpAspArgThrGluIleThrGlyGlnGlyAlaAlaPheArgArgLeuGlyAspValArgIleLeu

                                                                                      810
GCCGTCGTCGTGCCGAACCCGGTCGGTGTGATCGTGGACCGCGCGGGCACGGTGGTGCGCGGCAACTACGACGCGCAGACCGGGGTCCGG
AlaValValValProAsnProValGlyValIleValAspArgAlaGlyThrValValArgGlyAsnTyrAspAlaGlnThrGlyValArg

                                                                                      900
CGCCACCCGGTGTTCGACTACCAGGAGGCGTTCGCCGAGCAGGTCCCGCCCGTCACCGAGGCCGGCAACACCACGATCAGCGCGATCGTC
ArgHisProValPheAspTyrGlnGluAlaPheAlaGluGlnValProProValThrGluAlaGlyAsnThrThrIleSerAlaIleVal

                                                                                      990
ACGAACGTGCGGATGAGCCCCGTCGAGCTGAACCAGTTCGCCAAGCAGGTGCACAGTTCGATGCACCGCGGCATCCAGCCGTTCCACACC
ThrAsnValArgMetSerProValGluLeuAsnGlnPheAlaLysGlnValHisSerSerMetHisArgGlyIleGlnProPheHisThr

                                                                   SmaI              1080
GACATGGACGGCGACACGCTCTTCGCCGTCACCACCGACGAGATCGATCTGCCGACGACCCCGGGGTCGTCGCGCGGGCGGCTGTCGGTG
AspMetAspGlyAspThrLeuPheAlaValThrThrAspGluIleAspLeuProThrThrProGlySerSerArgGlyArgLeuSerVal

                                                                                     1170
AACGCGACCGCGCTCGGCGCGATCGCCTCCGAGGTGATGTGGGACGCCGTCCTCGAGGCCGGCAAGTAGGGGCCCGGCCCCGGAACAGGA
AsnAlaThrAlaLeuGlyAlaIleAlaSerGluValMetTrpAspAlaValLeuGluAlaGlyLys***

                                               BamHI 1218
GAGGAACGCTGTGGAGATCATCACCGCGACGGTGACGGGCACGGATCC
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FIG. 6. Nucleotide and amino acid sequences of the *nylC* gene. The nucleotide sequence presented is a 1,218-bp *Bgl*II-*Bam*HI fragment that includes the gene. The amino acid sequence deduced from the nucleotide sequence is shown below the nucleotide sequence. A Shine-Dalgarno sequence (GGAGG) and initiation (ATG) and termination (TAG) codons are boxed.

ment, it was confirmed that cell extracts of *E. coli* containing pUC19 produced no products from Nom1, Nom2, Z-AL3, AC2, and AL2 under the same reaction conditions. These results indicate that the *nylC* gene is included in the 1.2-kb *Bgl*II-*Bam*HI fragment (map position, 4.1- to 5.3-kb region on pOAD21) (Fig. 1).

Nucleotide sequence of the *nylC* gene. The nucleotide sequence of *nylC* was identified by the chain termination method by using dideoxynucleotide triphosphate. Figure 6 shows the sequence of the 1,218-bp *Bgl*II-*Bam*HI region included in plasmid pULC3. An open reading frame of 1,065 bp that begins at ATG, terminates at TAG, and encodes 355 amino acids was observed. The amino acid composition and N-terminal amino acid sequence of the EIII enzyme purified from the *E. coli* clone agreed well with the data deduced from the nucleotide sequence, although an additional N-terminal sequence obtained by proteolytic cleavage of the enzyme was observed (unpublished data). From these findings, we concluded that this open reading frame is the *nylC* gene. A Shine-Dalgarno sequence (GGAGG) complementary to the 3′ end of the 16S rRNA of *E. coli* was observed 4 bp upstream of the initiation codon.

The terminal *Bam*HI site in the 1,218-bp fragment was located 64 bp downstream of the termination codon. Sequencing of the downstream region of the *Bam*HI site revealed that the initiation (ATG) codon for the *nylB* gene was located 610 bp from the termination (TAG) codon of the *nylC* gene.

EIII enzyme encoded by parental plasmid pOAD2. We found that *E. coli* C600$r_K^-m_K^-$ harboring pNDH29 (14), a hybrid plasmid consisting of pBR322 and the *Hin*dIII A fragment from parental plasmid pOAD2, had activity toward Z-AL3 (data not shown). This indicates that a functional *nylC* gene is also retained in parental plasmid pOAD2 (Fig. 1). We determined the nucleotide sequence of the 1,218-bp *Bgl*II-*Bam*HI region of pNDH29 that includes the *nylC* gene of parental plasmid pOAD2 and found that two *nylC* genes from KI72 and KI725R1 were identical in the sequences of the 1,218-bp regions. When *Flavobacterium* sp. strain KI72 was inoculated onto an LB-AC2 plate (to check EI activity) or onto an LB–Z-AL3 plate (to check EIII activity), clear zones were formed around the cells by enzymatic hydrolysis of fine, insoluble substrates in the plates (Fig. 2B). These results also support the idea that the *nylC* gene in parental strain KI72 is functional.

DISCUSSION

A gene (*nylC*) that encodes a new type of nylon oligomer-degrading enzyme was found in the 4.2- to 5.3-kb region on the map of pOAD2 in the same orientation as the *nylB* gene (Fig. 1 and 5). Between the initiation (ATG) codon for the *nylB* gene and the termination (TAG) codon of the *nylC* gene, neither the inverted repeat sequences, a possible transcriptional terminator of the *nylC* gene, nor a sequence similar to the *E. coli* promoter consensus sequence was observed. This suggests that the *nylB* and *nylC* genes are expressed from a common promoter. Okazaki et al. have reported that a promoter functional in *E. coli* is located 6 kb upstream of the *nylB* gene (16), and two mRNAs with different sizes, i.e., 9 and 7 kb, were identified in *E. coli* harboring pNDH29 (containing the *Hin*dIII A fragment of pOAD2) by Northern (RNA) hybridization (23). It is likely that the two genes responsible for nylon oligomer metabolism constitute a single polycistronic unit in *E. coli*. In contrast to the close localization of these genes, the other nylon oligomer degradation gene (*nylA*) is located approximately 15 kb from the *nylB* and *nylC* regions on the same plasmid.

The ^{32}P-labeled *nylA* probe did not hybridize to the *nylC* region, even when Southern hybridization was done at 50°C (11). Sequence alignment by the method of Wirbur and Lipman (21) showed no significant homology among the *nylA*, *nylB*, and *nylC* genes. These results suggest that the three nylon oligomer-degradative enzymes evolved independently.

Although cell extracts of *Flavobacterium* sp. strains KI72, KI725R, and KI725 have hydrolytic activity toward Nom1 and Nom2, only the first two strains grow in Nom1 minimum medium. Several possibilities may explain the phenotypic differences between the KI725 and KI725R strains. Since activity toward Nom1 was enhanced in strains KI725R and KI7250R, it is conceivable that expression of the *nylC* gene is enhanced in these strains and the elevated enzyme activities made the cells Nom$^+$. However, the following possibilities could not be ignored: (i) KI725R strains may possess an additional nylon oligomer-degrading enzyme which is active toward a substrate included in Nom1, but KI725 has no degradative ability toward the substrate; (ii) nylon oligomer transport proteins are activated in KI725R strains; or (iii) the EIII proteins were altered by mutations in the coding region of the *nylC* gene by which the specific activities and/or substrate specificity of the enzyme were changed. The last possibility should be negligible, because the nucleotide sequences of the 1,218-bp *Bgl*II-*Bam*HI fragments containing

the *nylC* gene in the KI72 and KI725R1 strains are identical. Nucleotide sequencing of the whole pOAD2 plasmid is in progress, and analysis of alterations of pOAD2-encoded proteins among strains KI72, KI725, and KI725R1 would clarify the genetic steps which influence specific growth on various nylon oligomers.

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