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6-Aminohexanoate Oligomer Hydrolases from the Alkalophilic Bacteria *Agromyces* sp. Strain KY5R and *Kocuria* sp. Strain KY2[▽]

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Alkalophilic, nylon oligomer-degrading strains, *Agromyces* sp. and *Kocuria* sp., were isolated from the wastewater of a nylon-6 factory and from activated sludge from a sewage disposal plant. The 6-aminohexanoate oligomer hydrolases (NylC) from the alkalophilic strains had 95.8 to 98.6% similarity to the enzyme in neutrophilic *Arthrobacter* sp. but had superior thermostability, activity under alkaline conditions, and affinity for nylon-related substrates, which would be advantageous for biotechnological applications.

The biodegradation of unnatural synthetic compounds which have been released into the natural environment with the development of the chemical industry provides a suitable system for investigating how microorganisms evolve the enzymes essential for the degradation of such xenobiotic compounds. We have studied the degradation of by-products of the manufacture of nylon-6, namely, 6-aminohexanoate (Ahx) oligomers (nylon oligomers), by *Arthrobacter* sp. strain KI72 (formerly called a *Flavobacterium* sp. [see Addendum]) as a model system (9, 10). We found that three enzymes, the Ahx cyclic-dimer hydrolase (NylA) (6), the Ahx dimer hydrolase (NylB) (7, 12, 13), and the Ahx endo-type-oligomer hydrolase (NylC) (4, 11), are responsible for the degradation of the nylon oligomers (Fig. 1).

Nylon oligomers are discharged from nylon factories as an alkaline solution. However, the optimum pH for the growth of strain KI72 is approximately 7, and thus far, no alkalophilic, nylon oligomer-degrading strains have been isolated. Therefore, if such microorganisms could be isolated, they would be useful for the direct treatment of wastes from nylon-6 factories. In addition, comparative analyses for the responsible enzymes are expected to provide information on the evolutionary and functional divergence of these enzymes. In this paper, we report the isolation of alkalophilic, nylon oligomer-degrading bacteria, their genetic cloning, and the characterization of their nylon oligomer-degrading enzymes.

Isolation of alkalophilic nylon oligomer-degrading bacteria. The nylon oligomer mixture (NOM) (Toyobo Co., Tsuruga, Japan) used is a mixture of Ahx cyclic and linear oligomers. To obtain the cyclic-oligomer-enriched fraction used for bacterial screening, the NOM was extensively washed with hot water on filter paper to remove water-soluble linear oligomers. After being dried, washed NOM (NOM-W) was obtained. Thin-layer chromatography (TLC) analysis revealed that no free amino groups were detected by ninhydrin in NOM-W, which indicates the absence of the linear oligomer, while several spots appeared in the original NOM (Fig. 2C).

Microorganisms included in activated sludge from a sewage disposal plant (sample 1) and in wastewater from a nylon-6 factory (sample 2) were enriched in NOM10 medium (0.4% NOM-W, 0.2% $Na_3PO_4 \cdot 12H_2O$, 0.1% K_2HPO_4, 0.26% Na_2CO_3, 0.2% $NaHCO_3$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.5% NaCl, 0.01% yeast extract, pH 10). The cultures were diluted with sterilized water and plated on LB-NOM10 plates (LB plates containing 20 g/liter NOM-W, 100 mM carbonate buffer, pH 10). Nylon oligomer-degrading microorganisms were selected as colonies that form clear zones through the hydrolysis of insoluble NOM-W. After incubation at 30°C for 5 to 7 days, two strains (KY1 and KY2) and five strains (KY31, KY32, KY4, KY5R, and KY5S) were isolated from samples 1 and 2, respectively.

Enzymes responsible for the degradation of nylon oligomers in alkalophilic strains. To examine the structural similarity of the nylon oligomer-degrading enzymes among the isolated strains, cell extracts from each strain were analyzed by immunoblot analysis using antisera against NylA encoded on plasmid pOAD2 in *Arthrobacter* sp. strain KI72 ($NylA_{p2}$) and NylB encoded on pOAD2 ($NylB_{p2}$). Protein bands with similar electrophoretic mobilities were detected with the anti-$NylB_{p2}$ serum for all strains, suggesting that the NylB proteins in the alkalophilic strains were immunologically similar to $NylB_{p2}$. Similarly, NylA proteins were detected with the anti-$NylA_{p2}$ serum for all strains, except for KY5R and KY5S (data not shown).

NOM-W is a mixture of Ahx cyclic oligomers with various degrees of polymerization and is not appropriate for enzymatic analysis. To prepare an Ahx cyclic oligomer (for the NylC assay; degree of polymerization, >3), NOM-W (20 g/liter) dissolved in buffer A (20 mM phosphate buffer–10% glycerol, pH 7.3; 100 ml) was incubated with purified $NylA_{p2}$ enzyme (15 U/ml) at 30°C for 24 h to degrade the coexisting cyclic dimer, followed by extensive washing on filter paper with hot water. To confirm the absence of the cyclic dimer in this preparation, a portion was digested with either purified NylC from *Arthrobacter* plasmid pOAD2 ($NylC_{p2}$) (15 μmol/min [U/ml])

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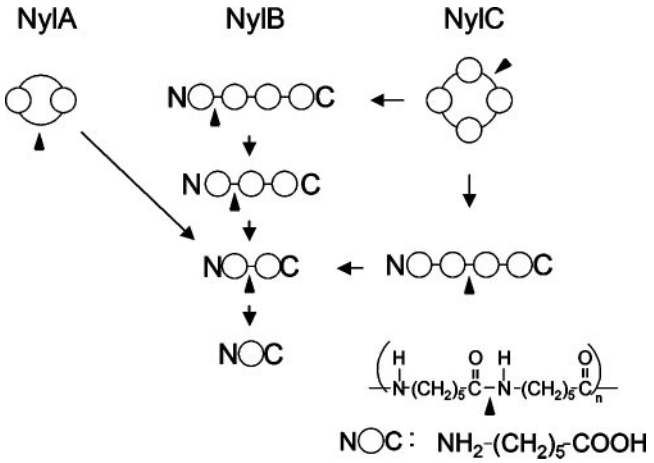

FIG. 1. Mode of degradation of nylon oligomers by the Ahx cyclic-dimer hydrolase (NylA), the Ahx dimer hydrolase (NylB), and the Ahx endo-type-oligomer hydrolase (NylC).

or $NylA_{p2}$ (15 U/ml), followed by TLC analysis. The $NylC_{p2}$-treated sample gave spots for Ahx and Ahx linear oligomers (Fig. 2C, slot 10), but no ninhydrin-reactive spots were detected even after the $NylA_{p2}$ treatment (Fig. 2C, slot 9). To obtain the Ahx cyclic dimer (for the NylA assay), NOM-W was incubated with $NylC_{p2}$ (15 U/ml), followed by crystallization from hot water. To confirm the absence of the cyclic oligomer in this sample, a portion was treated with either $NylA_{p2}$ or $NylC_{p2}$. The $NylA_{p2}$ digestion gave the Ahx linear dimer (Fig. 2C, slot 5), but $NylC_{p2}$ digestion gave no ninhydrin-positive spots (Fig. 2C, slot 6). The Ahx linear dimer (for the NylB assay) was chemically synthesized (7, 10).

After reaction of the cell extracts with the Ahx cyclic dimer (for the NylA assay) or Ahx linear dimer (for the NylB assay) for 3 h, reaction products were analyzed by TLC (12). The qualitative assay revealed the presence of NylA and NylB activities in all strains except KY5R and KY5S, which did not exhibit NylA activity. Moreover, no significant differences were observed in the activities at different pHs and temperatures (30°C/pH 10 and 60°C/pH 7.3) among the isolated strains and the neutrophilic strain KI72 (data not shown). When the Ahx cyclic oligomers (degree of polymerization, >3) were used as substrates (for the NylC assay), all strains tested produced Ahx and the Ahx linear dimer under the reaction conditions at 30°C/pH 7.3, suggesting that the cyclic oligomers are degraded to the linear oligomers, followed by conversion to Ahx (Fig. 2A). However, cell extracts from strains KY5R, KY5S, and KY2 produced reaction products even at 60°C and pH 7.3, while the cell extracts from KI72 and the other four strains produced no or smaller amounts of reaction products (Fig. 2B). These results suggest that NylC in KY5R, KY5S, and KY2 exhibited higher thermostability than NylC in the other strains.

Taxonomical study. We amplified the DNA regions containing the 16S rRNA gene with primers comprised of conserved sequences, cloned the amplified the fragments into *Escherichia coli*, and identified the nucleotide sequences. Strains KY5R and KY5S differed in their colony morphologies, but the 16S rRNA sequences were identical. A homology search against the DNA database using the BLAST program (http://blast.ddbj.nig.ac.jp/top-j.html) extracted 100 sequences; the 13 se-

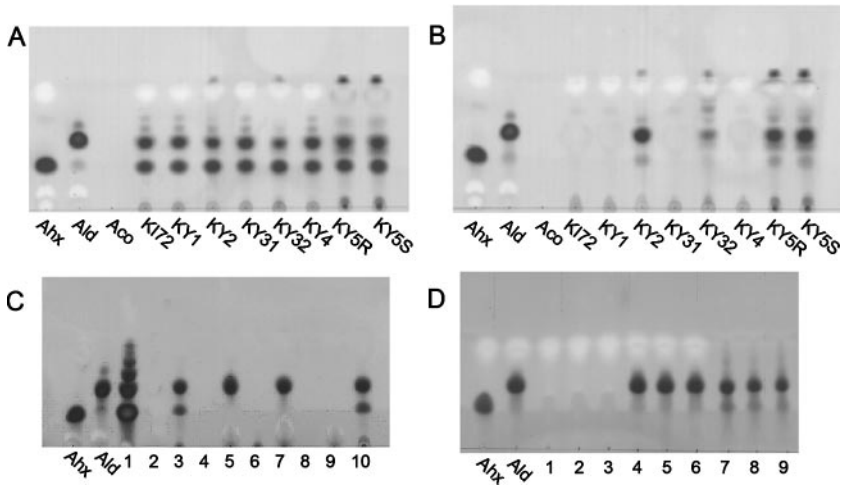

FIG. 2. TLC of various nylon oligomers and reaction products by nylon oligomer hydrolases. (A and B) Cell extracts (KI72, KY1, KY2, KY31, KY32, KY4, KY5R, and KY5S) were incubated for 3 h with 2 mg/ml of the Ahx cyclic oligomers (degree of polymerization, >3) (see panel C) at 30°C (A) or at 60°C (B) in 20 mM phosphate buffer containing 10% glycerol, pH 7.3 (buffer A). After a 1-μl aliquot was spotted, reaction products were developed and detected by spraying them with ninhydrin (12). Ahx, authentic Ahx; Ald, the authentic Ahx linear dimer; Aco, the Ahx cyclic oligomers. (C) The Ahx cyclic dimer and Aco (degree of polymerization, >3) were fractionated from the NOM by an enzyme reaction using purified $NylA_{p2}$ or $NylC_{p2}$. Samples at each preparation stage were analyzed by TLC (slots 1 to 10). Slot 1, unwashed NOM; slot 2, NOM-W (NOM washed with hot water on filter paper); slot 3, NOM-W digested with $NylC_{p2}$; slot 4, the purified Ahx cyclic dimer (used for NylA assay); slot 5, the Ahx cyclic dimer (sample 4) digested with $NylA_{p2}$; slot 6, the Ahx cyclic dimer (sample 4) digested with $NylC_{p2}$; slot 7, NOM-W digested with $NylA_{p2}$; slot 8, purified Aco (used for the NylC assay); slot 9, Aco (sample 8) digested with $NylA_{p2}$; slot 10, Aco (sample 8) digested with $NylC_{p2}$. (D) NylC purified from *E. coli* clones (0.1 mg/ml each) was incubated with a 10 mM concentration of the Ahx cyclic dimer, 10 mM Ald, and 2 mg/ml Aco at pH 7.3 at 30°C for 4 h, and reaction products were detected by TLC as described above. Slot 1, $NylC_{p2}$ plus the Ahx cyclic dimer; slot 2, $NylC_A$ plus the Ahx cyclic dimer; slot 3, $NylC_K$ plus the Ahx cyclic dimer; slot 4, $NylC_{p2}$ plus Ald; slot 5, $NylC_A$ plus Ald; slot 6, $NylC_K$ plus Ald; slot 7, $NylC_{p2}$ plus Aco; slot 8, $NylC_A$ plus Aco; slot 9, $NylC_K$ plus Aco.

Enzyme	Positions of amino acid residues															Kinetic parameters		Thermo-stability	Optimum pH
	36	41	50	60	62	111	122	130	137	225	230	231	257	262	354	k_{cat} (s^{-1})	K_m (mg/ml)		
$NylC_{p2}$	D	A	M	I	A	G	D	H	L	V	T	V	V	E	G	6.5±0.29	3.7±0.27	<40°C	6.5-7.5
$NylC_K$	A	V	T	V	S	S	G	Y	A	M	G	I	L	Q	A	11.7±0.16	0.44±0.022	<60°C	7.0-8.5
$NylC_A$	D	A	M	I	A	S	G	Y	A	M	T	V	V	E	G	11.1±0.14	0.49±0.025	<55°C	7.0-8.5

FIG. 3. Comparison of amino acid sequences and characterization of $NylC_{p2}$, $NylC_K$, and $NylC_A$. Amino acid residues are shown in the one-letter code. Fifteen residues differed between $NylC_{p2}$ and $NylC_K$. The five darkly shaded residues in both $NylC_K$ and $NylC_A$ were altered. Ten residues were unique to $NylC_K$ (lightly shaded). The NylC enzymes were purified and characterizations were compared. For quantitative NylC assay, the enzyme reactions were performed at 30°C using 1 mg/ml of the Ahx cyclic oligomer as a substrate in buffer A (see the text) (standard assay conditions), and an increase in the amino group was assayed using trinitrobenzene sulfonic acid (4, 6, 7). For the kinetic studies, NylC activity was assayed under standard assay conditions, except that various concentrations of the Ahx cyclic oligomer were used. Kinetic parameters (k_{cat} and K_m values) were evaluated by directly fitting the Michaelis-Menten equation to the data using the program GraphPad Prism, version 5 (Graphpad, San Diego, CA). Since the Ahx cyclic-oligomer substrate used for the enzyme assay includes Ahx cyclic oligomers with different degrees of polymerization, K_m values are expressed in mg/ml. Assuming that the Ahx cyclic oligomer is composed of the homogeneous Ahx cyclic tetramer (M_r = 448), the Ahx cyclic oligomer (1 mg/ml) corresponds to a 2.2 mM concentration of the Ahx cyclic tetramer. k_{cat} values are expressed as turnover numbers per α- or β-subunit (M_r = 36,000). For determination of thermostability, each NylC enzyme (1 mg/ml) was incubated at 30 to 75°C (5°C intervals) in buffer A for 30 min and residual activity was measured under standard assay conditions. Even after the heat treatment at the temperatures shown in the figure or lower temperatures, more than 90% of the enzyme activities were retained. To determine the optimum pH, the enzyme reaction was performed under standard assay conditions, except that pH was changed using 100 mM phosphate buffer (pHs 6.0, 6.5, 7.0, and 7.5), 100 mM barbital-HCl buffer (pHs 7.5, 8.0, 8.5, 9.0, and 9.5), or 100 mM carbonate buffer (pHs 9.5, 10.0, and 10.5). The pH range that exhibited more than 80% of the maximum activities are shown.

quences that had the highest scores (93.9 to 98.4%) were those of 16S rRNAs of *Agromyces* strains. Moreover, TLC analysis of the hydrolysate of peptidoglycan of the KY5R and KY5S strains revealed the presence 2,4-diaminobutyric acid, which is a typical characteristic of the genus *Agromyces* (18). On the basis of these results, we concluded that strains KY5R and KY5S were members of *Agromyces* sp. In addition, strain KY2 was identified as a *Kocuria* sp. based upon the high similarity (98.9 to 99.1%) to the other nine *Kocuria* strains (17). Since NylC in strains KY5R, KY5S, and KY2 possessed nylon oligomer-hydrolytic activities even at high temperatures and since the first two strains are phylogenetically identical, we focused further study on NylC from strains KY5R and KY2.

Cloning and sequencing of the *nylC* genes. In *Arthrobacter* sp. strain KI72, the *nylA*, *nylB*, and *nylC* genes are located on plasmid pOAD2 (5, 14, 15), while in *Agromyces* sp. strain KY5R and *Kocuria* sp. strain KY2, extrachromosomal DNA has not been detected by the conventional alkaline-extraction method (16). The 2.1-kb BglII fragment and 2.2-kb SacI fragment, which hybridized to the $nylC_{p2}$ probe, were cloned into plasmid pHSG299 (19) from the chromosomes of KY5R and KY2, respectively. From sequencing analyses, we found that the entire *nylC* gene was included in each cloned fragment. The *nylC* genes from *Agromyces* ($nylC_A$) and *Kocuria* ($nylC_K$) encoded polypeptides of 355 amino acid residues, which agrees with the size of $NylC_{p2}$. The three *nylC* genes had common initiation (ATG) and termination (TAG) codons and identical Shine-Dalgarno sequences (GGAGG). However, the $NylC_A$ and $NylC_K$ sequences had 5 and 15 amino acid alterations in their sequences compared to $NylC_{p2}$, respectively (Fig. 3).

To examine the characteristics of the three NylC proteins, the enzymes were purified from the cell extracts of *E. coli* JM109 (harboring a hybrid plasmid that highly expresses NylC) by ammonium sulfate fractionation (20%- to 35%-saturated fraction) and Hi-TrapQ–Sepharose column chromatography (0.15 to 0.25 M NaCl gradient). The purified NylC gave a single band on nondenaturing polyacrylamide gel electrophoresis (PAGE), indicating that the enzyme was purified to homogeneity. However, sodium dodecyl sulfate-PAGE analysis of the enzymes gave two bands (27 kDa [α-subunit] and 9 kDa [β-subunit]). To identify the processing sites in $nylC_A$ and $nylC_K$, we excised the protein band corresponding to the β-subunit from the PAGE gel. The 10 N-terminal residues obtained by protein sequencing were identical to those deduced from the nucleotide sequence starting from Thr267, demonstrating that $NylC_A$ and $NylC_K$ were subjected to specific cleavage at Asn266/Thr267, which has previously been identified as a cleavage site in $NylC_{p2}$ (4). The observed processing is a specific feature of the N-terminal nucleophile (Ntn) hydrolase family, in which cleavage is performed auto-catalytically to generate two subunits (1, 2, 3, 8).

Characterization of NylC. To examine their activities toward nylon-related substrates, the purified $NylC_A$, $NylC_K$, and $NylC_{p2}$ enzymes (0.1 mg/ml each) were incubated with the Ahx cyclic dimer, the Ahx linear dimer, or Ahx cyclic oligomers (degree of polymerization, >3) at 30°C for 4 h and reaction products were analyzed by TLC (Fig. 2D). The three enzymes produced Ahx from Ahx cyclic oligomers (the NylC-specific substrate) (Fig. 2D, slots 7 to 9) but gave no detectable amounts of reaction products from either the Ahx cyclic dimer (the NylA-specific substrate) (Fig. 2D, slots 1 to 3) or the Ahx linear dimer (the NylB-specific substrate) (Fig. 2D, slots 4 to 6). However, it should be noted that the NylC enzymes have trace activities for the Ahx linear dimer (ca. 1% of the specific activity for Ahx cyclic oligomers), since reaction mixtures using 100 times more NylC samples (10 mg/ml) gave similar ratios of conversion of the Ahx linear dimer to Ahx obtained with 0.1-mg/ml NylB samples. These results indicate that $NylC_A$ and $NylC_K$ are basically similar to $NylC_{p2}$ in specificity for nylon-related compounds. However, as described below, the $NylC_A$ and $NylC_K$ enzymes exhibited different levels of thermostability, optimum pHs, and kinetic parameters.

Thermostability. $NylC_{p2}$ was stable only up to 40°C after a 30-min incubation. In contrast, $NylC_A$ and $NylC_K$ retained approximately 90% of their enzyme activity even after incubation at 55°C and 60°C, respectively (Fig. 3).

Effect of pH on enzyme activity. $NylC_{p2}$ had an optimum pH at approximately 7.0, whereas the pH activity curves of $NylC_A$ and $NylC_K$ were shifted 0.5 to 1.0 points into the alkaline range. Under standard assay conditions (pH 7.3) (see the legend to Fig. 3), $NylC_{p2}$ had a specific activity of 10.6 U/mg, while $NylC_A$ (17.9 U/mg) and $NylC_K$ (18.9 U/mg) possessed higher enzyme activities.

Kinetic study. To compare the kinetic parameters, enzyme assays were performed at pH 7.3, at which pH all NylC samples possess more than 90% of the activity observed at the optimum pH. $NylC_A$ (K_m = 0.49 mg/ml) and $NylC_K$ (K_m = 0.44 mg/ml) possessed higher affinities for the Ahx cyclic-oligomer substrate than $NylC_{p2}$ (K_m = 3.7 mg/ml), resulting in 13-fold ($NylC_A$) and 15-fold ($NylC_K$) greater k_{cat}/K_m values than that of $NylC_{p2}$ (Fig. 3).

Considering the above observations, we suggest that at least one among the five alterations in $NylC_A$ (S111, G122, Y130, A137, M225) contributes to the increase in thermostability, the shift of the pH activity profile to the alkaline range, and the increase in the affinity for nylon-related substrates. In addition, at least one of the 10 alterations in $NylC_K$ (A36, V41, T50, V60, S62, G230, I231, L257, Q262, A354) is estimated to contribute to the further increase in thermostability (Fig. 3).

In conclusion, we found that the NylC enzymes obtained from alkalophilic *Agromyces* and *Kocuria* strains possess higher thermostability, greater activity under alkaline conditions, and greater affinity for nylon-related substrates than NylC from the neutrophilic *Arthrobacter* strain. Therefore, the observed characteristics of the new NylC proteins should provide great advantages in biotechnological applications.

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ADDENDUM

Strain KI72 had been classified as a *Flavobacterium* sp. based upon ordinary Gram staining and physiological tests (14). However, reclassification on the basis of its chemotaxonomic characteristics (20) and 16S rRNA sequences (this study) revealed that strain KI72 should be classified as an *Arthrobacter* sp.

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