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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Metabolic pathway of 6-aminohexanoate in the nylon oligomer-degrading bacterium *Arthrobacter* sp. KI72: identification of the enzymes responsible for the conversion of 6-aminohexanoate to adipate

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Abstract

Arthrobacter sp. strain KI72 grows on a 6-aminohexanoate oligomer, which is a by-product of nylon-6 manufacturing, as a sole source of carbon and nitrogen. We cloned the two genes, *nylD*$_1$ and *nylE*$_1$, responsible for 6-aminohexanoate metabolism on the basis of the draft genomic DNA sequence of strain KI72. We amplified the DNA fragments that encode these genes by polymerase chain reaction using a synthetic primer DNA homologous to the 4-aminobutyrate metabolic enzymes. We inserted the amplified DNA fragments into the expression vector pColdI in *Escherichia coli*, purified the His-tagged enzymes to homogeneity, and performed biochemical studies. We confirmed that 6-aminohexanoate aminotransferase (NylD$_1$) catalyzes the reaction of 6-aminohexanoate to adipate semialdehyde using α-ketoglutarate, pyruvate, and glyoxylate as amino acceptors, generating glutamate, alanine, and glycine, respectively. The reaction requires pyridoxal phosphate (PLP) as a cofactor. For further metabolism, adipate semialdehyde dehydrogenase (NylE$_1$) catalyzes the oxidative reaction of adipate semialdehyde to adipate using NADP$^+$ as a cofactor. Phylogenic analysis revealed that NylD$_1$ should be placed in a branch of the PLP-dependent aminotransferase sub III, while NylE$_1$ should be in a branch of the aldehyde dehydrogenase superfamily. In addition, we established a NylD$_1$/NylE$_1$ coupled system to quantify the aminotransferase activity and to enable the conversion of 6-aminohexaoate to adipate via adipate semialdehyde with a yield of > 90%. In the present study, we demonstrate that 6-aminohexanoate produced from polymeric nylon-6 and nylon oligomers (i.e., a mixture of 6-aminohexaoate oligomers) by nylon hydrolase (NylC) and 6-aminohexanoate dimer hydrolase (NylB) reactions are sequentially converted to adipate by metabolic engineering technology.

Keywords 6-Aminohexanoate · 4-Aminobutyrate · Aminotransferase · Aldehyde dehydrogenase · Bioconversion · Adipate

Introduction

Nylons are synthetic polymers that contain recurring amide groups in their main polymer chains. Due to their high strength, elasticity, and chemical and thermal resistance, nylons have been used for the production of various fibers and plastics. Particularly, nylon-6 (PA6) and nylon-66 (PA66) account for approximately 90% of the total production of synthetic polyamides. PA6 is composed of a single monomeric unit of 6-aminohexaoate (Ahx), whereas two monomeric units, hexamethylenediamine and adipate, are alternatively combined in PA66 (Travis 1998; McIntyre 2005). PA6 is industrially obtained by the ring cleavage polymerization of ε-caprolactam. A major route of ε-caprolactam production involves oxime formation with NH_2OH (prepared from NH_3 and H_2O_2) and a Beckmann rearrangement using cyclohexanone as the starting material in the presence of a strong acid, which generates large amounts of salts as by-products (Travis 1998). The chemical reaction responsible for adipate

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production also requires high temperature and pressure and produces hazardous substances such as nitrous oxide as a by-product (McIntyre 2005). Attempts to develop the bio-based production of adipate (Chen and Nielsen 2013; Draths and Frost 1994; Polen et al. 2013) and Ahx (Sattler et al. 2014; Turk et al. 2016) using synthetic biology and metabolic engineering have been made as an approach for the construction of a sustainable production system. Moreover, environmental considerations suggest that nylon recycling should be mandatory. However, most nylon wastes are currently disposed of by burning or dumping, although the chemical conversion of PA6 to ε-caprolactam has been reported (Chen et al. 2010; Iwaya et al. 2006). Biochemical studies on the relevant enzymes are important to achieve the biotechnological production and recycling of nylons.

Arthrobacter sp. KI72 is a bacterium that can grow on an Ahx oligomer (designated as "nylon oligomer") as the sole carbon and nitrogen source (Okada et al. 1983). The strain harbors three different plasmids, pOAD1, pOAD2, and pOAD3 (Kato et al. 1995). Previous biochemical studies have revealed that three enzymes NylA, NylB, and NylC encoded on pOAD2 are responsible for the degradation of the Ahx oligomer to monomers (Negoro 2000) (Fig. 1a). The Ahx-cyclic dimer hydrolase (NylA; EC3.5.2.12), a member of the amidase signature hydrolase family, specifically hydrolyzes one of the two amide bonds in the Ahx-cyclic dimer to generate an Ahx linear dimer (Yasuhira et al. 2010). Ahx dimer hydrolase (NylB; EC3.5.1.46), a member of the penicillin-recognizing family of serine-reactive hydrolases, hydrolyzes the Ahx oligomers by an exo-type mechanism (Negoro et al. 2005, 2007). The Ahx oligomer hydrolase (NylC; EC3.5.-.-) degrades the Ahx-cyclic and Ahx-linear oligomers with a degree of polymerization greater than three by an endo-type mechanism (Negoro 2000; Yasuhira et al. 2007a; Negoro et al. 2012). The thermostabilized NylC protein variant degrades thin-layered PA6 (thickness 0.26 μm) almost completely at a constant reaction rate (Nagai et al. 2014).

The compound 4-aminobutyrate (γ-aminobutyrate: GABA), which contains shorter methylene chains than Ahx, is a non-protein amino acid produced by the decarboxylation of L-glutamate by glutamate decarboxylase in most eukaryotic and prokaryotic organisms. GABA plays an important role as a neurotransmitter in mammalian cells (Macdonald and Olsen 1994). Stress in plants initiates a calmodulin-dependent signal transduction pathway, in which increased cytosolic Ca^{2+} activates glutamate decarboxylase (Shelp et al. 1999). The degradation of GABA begins with its transamination to succinate semialdehyde (SSA) catalyzed by 4-aminobutyrate aminotransferase (EC 2.6.1.19) (Schneider et al. 2002) (Fig. 1b). The SSA is subsequently oxidized to succinate by $NAD(P)^+$-dependent succinate semialdehyde dehydrogenase (EC 1.2.1.16) (Fig. 1e) or reduced to γ-hydroxybutyrate by succinate semialdehyde reductase (EC 1.1.1.79) (Kockelkorn and Fuchs 2009). However, the enzymes responsible for the metabolisms of Ahx (containing longer methylene chains than GABA) have not been identified so far.

We have recently reported the draft genomic sequence of *Arthrobacter* strain KI72 (Takehara et al. 2017). In the present study, we screened two putative genes ($nylD_1$ and $nylD_2$) for the production of 6-aminohexanoate aminotransferase and 20 putative genes ($nylE_1$–$nylE_{20}$) for the production of adipate semialdehyde dehydrogenase from a homology search of the genomic sequence of strain KI72. We selected two genes ($nylD_1$ and $nylE_1$) from the phylogenic analysis, purified and characterized their gene products, and established a coupled reaction system composed of $NylD_1$ and $NylE_1$that enables the continuous monitoring of the amino transferase activity and complete conversion of 6-aminohexanoate to adipate.

Materials and methods

Chemicals

6-Aminohexanoate (Ahx), pyridoxal phosphate (PLP), and L-glutamate were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Adipate, succinate, pyruvate, glyoxylate, L-alanine, and glycine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). NAD^+, and NADPH were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). 4-Aminobutyrate (GABA) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). α-Ketoglutarate (α-KG) was purchased from Sigma-Aldrich Co. LLC (St. Louis, USA). Adipate semialdehyde (ASA) and succinate semialdehyde (SSA) were chemically synthesized in our laboratory. The details are described in the supplementary text. The structure of the synthesized compounds was confirmed by an NMR analysis. We stored the ASA and SSA samples by freezing them at − 20 °C and prepared the substrate solution just before use.

Microorganisms, DNA preparation, and plasmid construction

The bacterial strains, plasmids, and enzymes used in this study are described in Table S1. *Arthrobacter* sp. KI72 strains (NBRC 14590, National Institute of Technology and Evaluation, Japan) were grown at 30 °C in 100 ml of LB medium. Whole genome DNA was prepared from strain KI72 by the conventional phenol/chloroform extraction method (Sambrook and Russell 2001). $NylD_1$ and $NylE_1$ were designed to be expressed as the His-Tag fused proteins. The

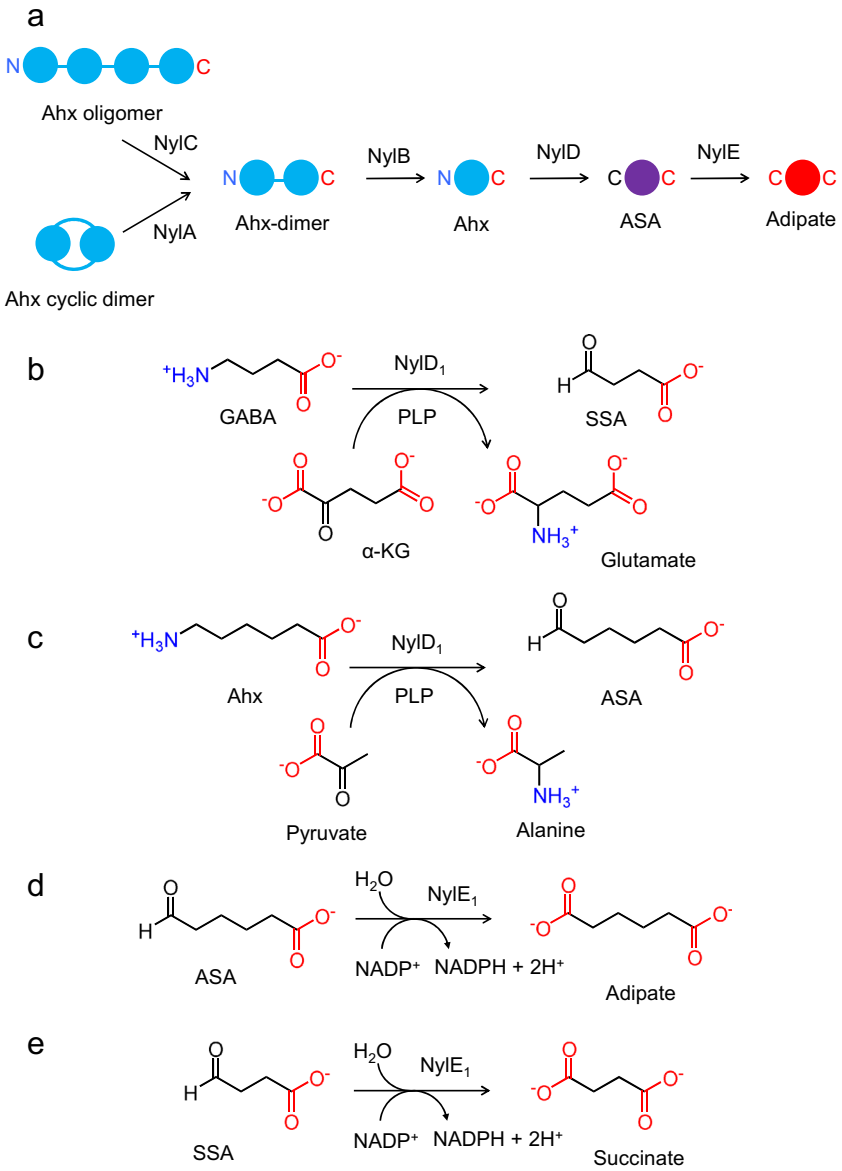

Fig. 1 Enzymatic degradation of 6-aminohexanoate and its oligomers in *Arthrobacter* sp. KI72. **a** Five enzymes responsible for the degradation of 6-aminohexanoate-oligomer to adipate are schematically illustrated. NylA, 6-aminohexanoate-cyclic dimer hydrolase; NylB, 6-aminohexanoate dimer hydrolase, NylC, endo-type 6-aminohexanoate oligomer hydrolase, or nylon hydrolase; NylD, 6-aminohexanoate aminotransferase; NylE, adipate semialdehyde dehydrogenase. The monomeric units of 6-aminohexanoate, adipate semialdehyde, and adipate are illustrated as *light blue*, *purple*, and *red closed circles*, respectively. Terminal amino N, carboxyl C, and aldehydic C are shown as *light blue*, *red*, and *black letters*, respectively. **b**, **c** Reactions with GABA (**b**) or Ahx (**c**) catalyzed by $NylD_1$ are shown. **d**, **e** Reactions from ASA (**d**) or SSA (**e**) catalyzed by $NylE_1$ are shown

$NylD_1$ and $NylE_1$ genes were amplified from *Arthrobacter* sp. KI72 genomic DNA by PCR using the primers described in Table S2. The amplified fragments were inserted into a pColdI expression vector (Takara Bio Inc. Shiga, Japan), and the ligated DNA was used to transform *Escherichia coli* BL21 (DE3) cells. The resulting plasmids, pCold-$NylD_1$ and pCold-$NylE_1$, were constructed for the expression of the recombinant proteins. *E. coli* strains harboring the hybrid plasmid were grown at 37 °C in LB medium containing ampicillin (100 μg/ml). Plasmid DNA was prepared by the conventional alkaline extraction method (Sambrook and Russell 2001).

Phylogenic analysis

The phylogenetic tree was constructed using the Clustal W programs (http://www.ebi.ac.uk/tools/clustalw2, Larkin et al. 2007).

Expression of *nylD*$_1$ and *nylE*$_1$ genes in *E. coli* and enzyme purification

To purify the His-tagged $NylD_1$ and $NylE_1$ enzymes, *E. coli* cells obtained by centrifugation (14,500×*g* for 10 min, 4 °C) were lysed by sonication (20 kHz, 30 s × 12 times) in 10 mL

of 100 mM potassium phosphate buffer (KPB, pH 7.0). Cell extracts obtained by centrifugation were used as crude enzyme solutions. The enzymes were purified from the supernatant using TALON metal affinity resin (Takara Bio USA, Inc. California, USA) following the manufacturer's instructions. Following purification, the enzymes were identified by SDS-PAGE, and the protein bands were visualized by staining with Coomassie Blue. The fraction containing $NylD_1$ and $NylE_1$ was dialyzed overnight against 100 mM KPB (pH 7.0). The protein concentration was determined with the Bradford assay using bovine serum albumin as the standard.

Enzyme assays

For the quantitative assay of the semialdehyde dehydrogenase (NylE) activity, the enzyme was incubated at 30 °C with 0.1 mM synthetic semialdehyde (ASA or SSA) and 0.1 mM $NADP^+$ in 100 mM KPB (pH 7.8). The reaction was initiated by the addition of $NylE_1$, and the rate of the oxidative reaction was monitored as the change in the NADPH concentration, as indicated by the absorbance at 340 nm (A_{340}) (ε = 6200 M^{-1} cm^{-1}) using a spectrometer (V-730_{BIO}, Jasco, Japan).

For the qualitative detection of the aminotransferase (NylD) activity by thin layer chromatography (TLC), the enzyme was incubated at 30 °C with 5 mM of the amino donor (Ahx or GABA), 5 mM of the amino acceptor (α-KG), and 0.1 mM PLP in 100 mM KPB (pH 7.8). One microliter of the reaction mixture was spotted on a thin-layer silica gel plate (TLC silica gel 60, Merck Millipore) and developed with solvent I (1-propanol:ethyl acetate:water:25% ammonia solution = 4.49:0.75:4.49:0.28). The reaction product on the TLC plate was then detected by spraying it with a 0.2% ninhydrin solution in butanol saturated with water as described previously (Negoro et al. 2005).

For quantitation of the Ahx aminotransferase activity, a $NylD_1$ reaction was performed in the presence of a great excess of $NylE_1$ enzyme activity (i.e., the $NylD_1$/$NylE_1$ coupled system). $NylD_1$ and $NylE_1$ were incubated at 30 °C with 0.2 mM of the amino donor (Ahx), 0.25 mM of the amino acceptor (α-KG, pyruvate, or glyoxylate), 0.1 mM PLP, and 0.25 mM $NADP^+$ in 100 mM KPB (pH 7.8) (i.e., the standard assay conditions for the coupled system). The rate of the reaction was monitored at A_{340}. Aminotransferase activity was calculated by subtracting the background level of the activity (without $NylD_1$) from the activity (in the coupled system) (see Fig. 7). For a reaction using a higher concentration of Ahx in the TLC analysis, the enzyme reactions were performed in 1.0 mL of a reaction mixture containing 2 mM Ahx, 2.5 mM of the amino acceptor, 0.1 mM PLP, 2.5 mM $NADP^+$, and 100 mM KPB buffer (pH 7.8). Aliquots of 30 μl were sequentially removed, and the reaction products were visualized by TLC. The NADPH concentration was determined from the A_{340}.

Results

Determination of the genes responsible for 6-aminohexanoate metabolism

We planned to clone the genes responsible for Ahx metabolism in *Arthrobacter* sp. strain KI72, assuming that the Ahx is converted to adipate by enzymes analogous to those involved in GABA metabolism (Fig. 1). On the basis of the protein assignment performed for the draft genomic sequence composed of 105 contig sequences [4,568,574 base pairs (bp) in total length] (Takehara et al. 2017), we screened two putative enzymes ($NylD_1$ and $NylD_2$) that were homologous to the GABA aminotransferases on the genome of *Arthrobacter* sp. KI72 (Fig. 2). We also identified 20 putative semialdehyde dehydrogenases ($NylE_1$–$NylE_{20}$), which should be classified in the aldehyde dehydrogenase (ALDH) superfamily (Fig. 3). In the following sections, we describe the phylogenic relationship of these enzymes.

Phylogenic relationship of NylD

PLP-dependent aminotransferases have been phylogenetically classified in four classes (Alexander et al. 1994, Mehta et al. 1993) but have recently been further divided into five subgroups (Hwang et al. 2005). Subgroups I and II include aromatic and aspartic acid transaminases, subgroup IV includes the branched-chain transaminases, and subgroup V includes the serine and histidinol phosphate transaminases. Phylogenic analyses revealed that $NylD_1$ and $NylD_2$ should be placed in subgroup III, which includes GABA aminotransferase (EC 2.6.1.19), ornithine aminotransferase (OAT; EC2.6.1.13), ω-amino acid-pyruvate aminotransferase (OAPT) (EC2.6.1.18), acetylornithine aminotransferase (ACOAT) (EC2.6.1.11), 7,8-diaminopelargonic acid aminotransferase (DAPAT) (EC2.6.1.62), 2,2-dialkylglycine decarboxylase (DGD) (EC4.1.1.64), and glutamate-1-semialdehyde aminomutase (GSAT; EC5.4.3.8) (Table S3). Both $NylD_1$ and $NylD_2$ are phylogenetically related to GABA aminotransferase. $NylD_1$ had 95.0% overall homology to GABA aminotransferase (GabT) from *Arthrobacter aurescens* TC1 (Ara-GABT), which degrades *s*-triazine-like pesticide compounds (Strong et al. 2002; Fig. 2). In addition, GABA aminotransferase from *Rhodococcus* (Rho-GABT), *Mycobacterium* (Myc-GABT), *Streptomyces griseoruber* (Str-GABT), and *E. coli* (Eco-GABT) had 73.0, 62.5, 54.6, and 42.8% homology to $NylD_1$, respectively. In contrast, $NylD_2$ had a relatively large sequence disparity from $NylD_1$ (49.8% homology), although $NylD_1$ and $NylD_2$ are isozymes from the same bacterial strain. Ornithine aminotransferase from *E. coli* (Eco-OAT) had a different substrate specificity and a much greater sequence disparity in the phylogenic relationship (34.9% homology to $NylD_1$).

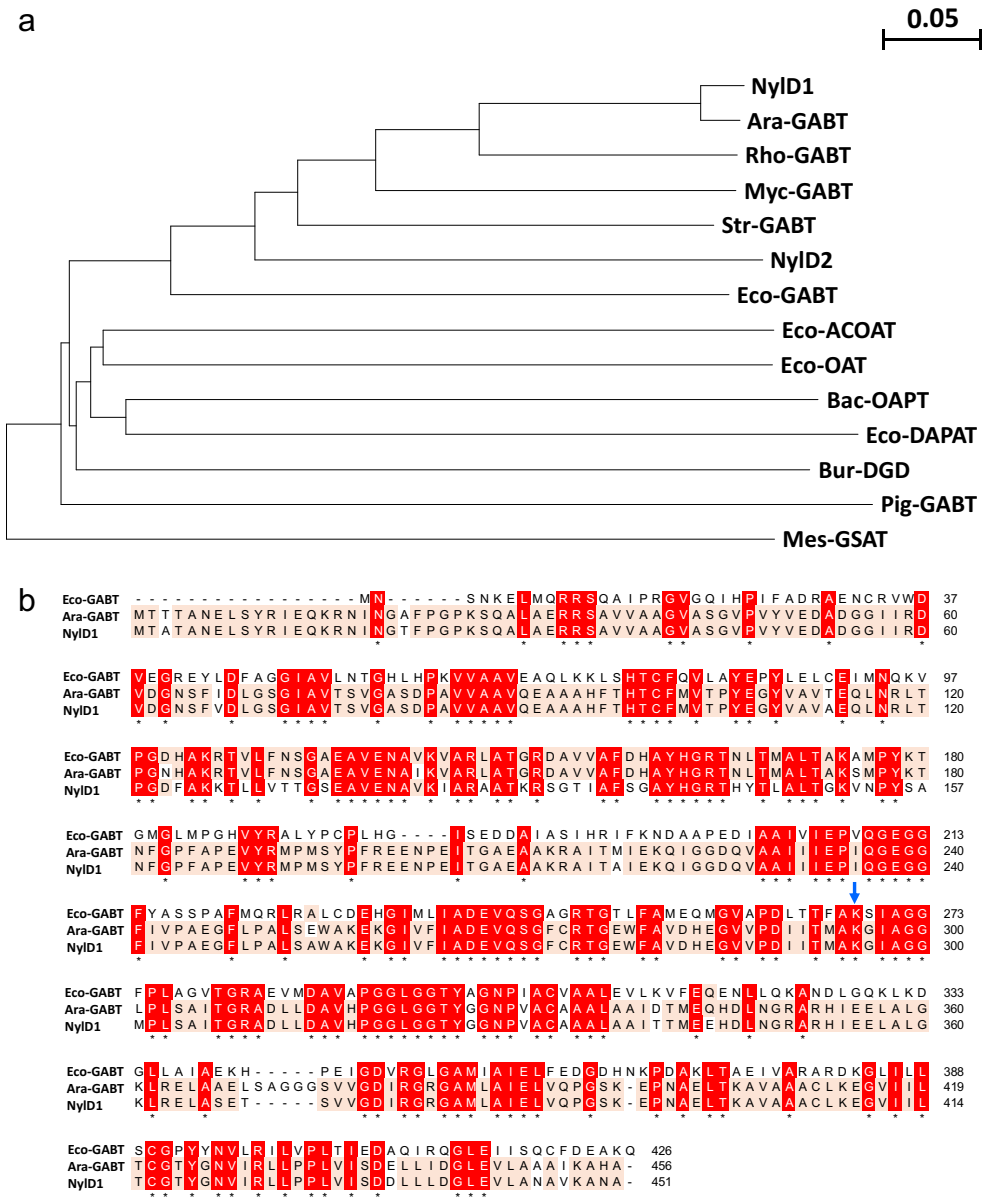

Fig. 2 Phylogenic analysis and alignment of the amino acid sequences of NylD. **a** A phylogenetic tree showing the evolutionary relationships among the 6-aminohexanoate aminotransferase ($NylD_1$, $NylD_2$), and the PLP-dependent aminotransferases classified as subgroup III are shown: Ara-GABT, GABA aminotransferase from *Arthrobacter aurescens*; Rho-GABT, GABA aminotransferase from *Rhodococcus erythropolis*; Myc-GABT, GABA aminotransferase from *Mycobacterium smegmatis*; Str-GABT, GABA aminotransferase from *Streptomyces griseoruber*; Eco-GABT, GABA aminotransferase from *Escherichia coli*; Eco-ACOAT, acetylornithine aminotransferase from *E. coli*; Eco-OAT, ornithine aminotransferase from *E. coli*; Bac-OAPT, ω-amino acid-pyruvate aminotransferase from *Bacillus anthracis*; Eco-DAPAT, 7,8-diaminopelargonic acid aminotransferase from *E. coli*; Bur-DGD, 2,2-dialkylglycine decarboxylase from *Burkholderia cepacia*; Pig-GABT, GABA aminotransferase from pig; Mes-GSAT, glutamate-1-semialdehyde aminomutase from *Mesorhizobium* sp. $NylD_1$ and $NylD_2$ are identified on different loci: $NylD_1$ on contig 23 and $NylD_2$ on contig 22. A detailed description of enzymes, protein ID, and the references used for the phylogenic analysis are shown in Table S3. **b** The amino acid sequence of $NylD_1$ was aligned with the sequences of Ara-GABT and Eco-GABT. The Lys residue responsible for PLP binding (marked by a *blue* arrow) was conserved among the three enzymes. Amino acid residues are shown as *one-letter code*

Phylogenic relationships of NylE

The aldehyde dehydrogenase (ALDH) superfamily comprises diverse protein families distributed among various eukaryotic and prokaryotic organisms (Perozich et al. 1999). Approximately 20,000 genes for the predicted enzymes have been phylogenetically classified into at least 13 families in the ALDH superfamily (Sophos and Vasiliou 2003). The phylogenic analyses suggest that $NylE_2$, $NylE_4$, $NylE_5$, and $NylE_8$ should be classified in the succinate semialdehyde

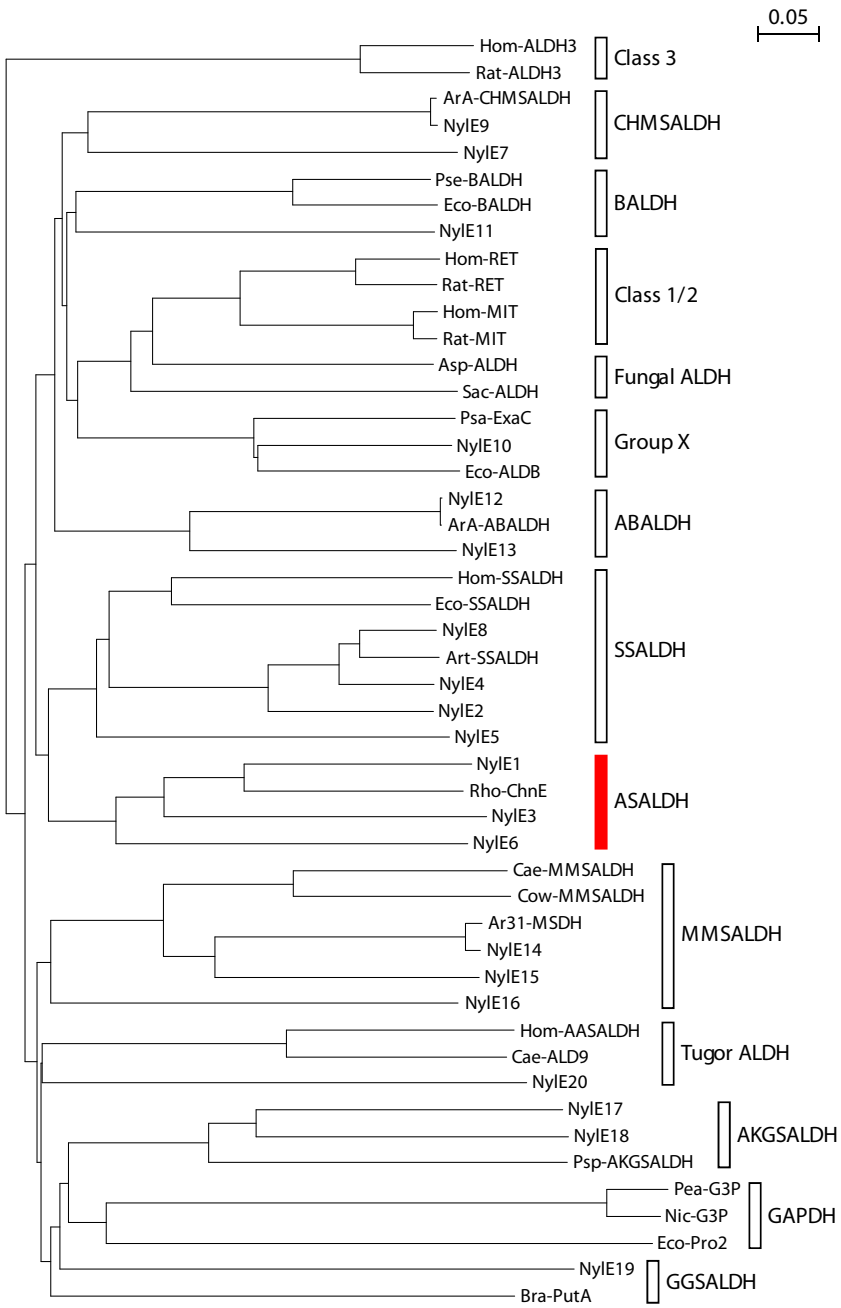

Fig. 3 Phylogenic analysis of NylE. A phylogenetic tree showing the evolutionary relationships among the 20 putative aldehyde dehydrogenases ($NylE_1$–$NylE_{20}$) identified in the *Arthrobacter* strain KI72 and typical enzymes in the aldehyde dehydrogenase superfamily (Hwang et al. 2005; Taniyama et al. 2012). $NylE_1$–$NylE_{20}$ were identified on the various loci on the genome of *Arthrobacter* sp. KI72: $NylE_1$, contig 1; $NylE_2$, contig 15; $NylE_3$, contig 4; $NylE_4$, contig 6; $NylE_5$, contig 39; $NylE_6$, contig 49; $NylE_7$, contig 4; $NylE_8$, contig 22; $NylE_9$, contig 4; $NylE_{10}$, contig 23; $NylE_{11}$, contig 5; $NylE_{12}$, contig 5; $NylE_{13}$, contig 23; $NylE_{14}$, contig 26; $NylE_{15}$, contig 62; $NylE_{16}$, contig 2; $NylE_{17}$, contig 7; $NylE_{18}$, contig 25; $NylE_{19}$, contig 7; and $NylE_{20}$, contig 5. Detailed description of enzymes, protein I.D., and references used for the phylogenic analysis are shown in Table S4

dehydrogenase (SSALDH) family, which has been identified in various organisms (Fig. 3, Table S4). In contrast, $NylE_1$ had the highest homology (63.5%) with adipate semialdehyde (6-oxohexanoate) dehydrogenase (the *chnE* gene from *Rhodococcus*, Rho-ChnE) (Fig. S2; Iwaki et al. 1999). We suggest that $NylE_1$ should be placed with $NylE_3$, $NylE_6$, and ChnE at a new branch designated the "adipate semialdehyde dehydrogenase (ASALDH) subfamily" distinguished from the other subfamilies (Fig. 3). As described below, functional analysis of the purified enzyme demonstrates that $NylE_1$ is specific for Ahx metabolism rather than GABA metabolism.

The remaining aldehyde dehydrogenases, $NylE_{12}$ and $NylE_{13}$, should be placed in the γ-aminobutyraldehyde dehydrogenase (ABALDH) subfamily, $NylE_{17}$ and $NylE_{18}$ in the α-ketoglutarate semialdehyde dehydrogenase (AKGSALDH) subfamily, $NylE_7$ and $NylE_9$ in the 5-carboxymethyl-2-hydroxymuconate semialdehyde dehydrogenase (CHMSALDH) subfamily, $NylE_{11}$ in the betaine aldehyde dehydrogenase (BALDH) subfamily, $NylE_{14}$ and $NylE_{15}$ in the methylmalonyl semialdehyde dehydrogenase (MMSALDH) subfamily, $NylE_{20}$ in the Tugor ALDH subfamily, and $NylE_{19}$ in the γ-glutamyl semialdehyde

(GGSALDH) subfamily. $NylE_{10}$ should be classified in a "Group X" subfamily as suggested by Taniyama et al. (2012). Regarding $NylE_1$–$NylE_{20}$, no enzymes classified in the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) subfamily were found.

Cloning, expression, and purification of $NylD_1$ and $NylE_1$

To quantitatively compare the catalytic function of the various NylD and NylE enzymes, efficient gene expression followed by the purification of the gene product and construction of an accurate enzyme assay system to analyze the catalytic activities are required. Further studies were focused on the enzymes $NylD_1$ and $NylE_1$, and we evaluated their catalytic functions to determine the physiological roles of these enzymes.

We amplified the DNA fragments containing the *nylD*$_1$ gene by PCR using strain KI72 genomic DNA as a template and synthetic oligonucleotide primers (Table S2). We expressed $NylD_1$ as His-tagged proteins using the pColdI expression vector (Fig. 4a). Similarly, we cloned and expressed $NylE_1$ as the His-tagged enzyme (Fig. 4b). Nucleotide sequencing of the DNA fragments cloned in the pCold-$NylD_1$ or pCold-$NylE_1$ vectors revealed that no mutations were integrated during the PCR and cloning processes. We purified the His-tagged enzymes from the cell extracts of the *E. coli* clones. The purified enzymes produced a single protein band on SDS-polyacrylamide gel electrophoresis corresponding to molecular weights of approximately 47 kDa ($NylD_1$) and 49 kDa ($NylE_1$) that corresponded well to the theoretical values expected from the nucleotide sequences (Fig. 4c).

$NylD_1$ We performed the enzyme reaction using an amino donor (Ahx or GABA) and amino acceptor (α-KG) to confirm the catalytic function of the enzyme. We identified the spot corresponding to L-glutamate in the reaction mixture by TLC (Fig. 5a). Although the intensity of the spot was weak, the presence of the expected reaction product demonstrates that $NylD_1$ has aminotransferase activity either for Ahx or GABA. In a reaction using glyoxylate as the amino acceptor, the conversion of Ahx to glycine was approximately 50% after a 3-h reaction, as indicated by the intensity of the spots identified by ninhydrin, which were found to be nearly at a similar level (Fig. 5e). Thus, the increase in the reaction apparently stopped due to the reverse reaction of $NylD_1$. However, as stated below, we found that the $NylD_1$ reaction coupled with $NylE_1$ significantly enhanced the conversion (Fig. 5b–d).

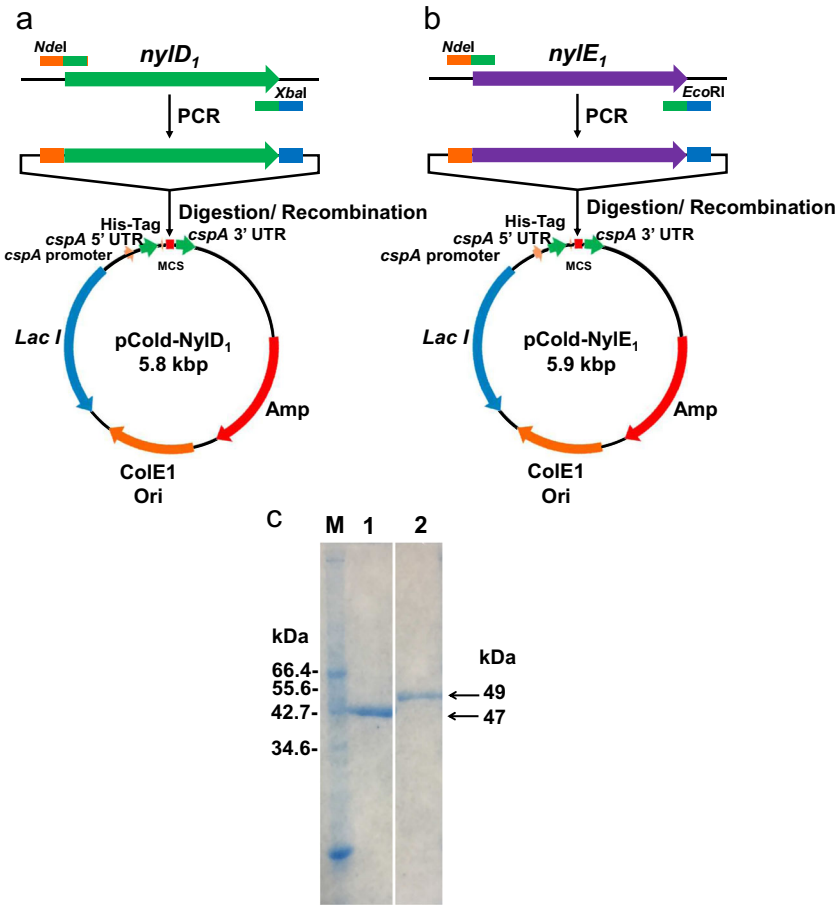

Fig. 4 Construction of hybrid plasmids expressing $NylD_1$ and $NylE_1$. **a**, **b** DNA fragments coding for *nylD*$_1$ (**a**) or *nylE*$_1$ (**b**) genes were amplified by PCR using the primers described in Table S2. The amplified fragments were inserted into the vector pColdI. The enzymes were expressed as the His-tag fused protein. **c** His-tagged $NylD_1$ and $NylE_1$ were analyzed by SDS-polyacrylamide (10%) gel electrophoresis. Slot 1, $NylD_1$; slot 2, $NylE_1$; slot M, molecular weight standard proteins

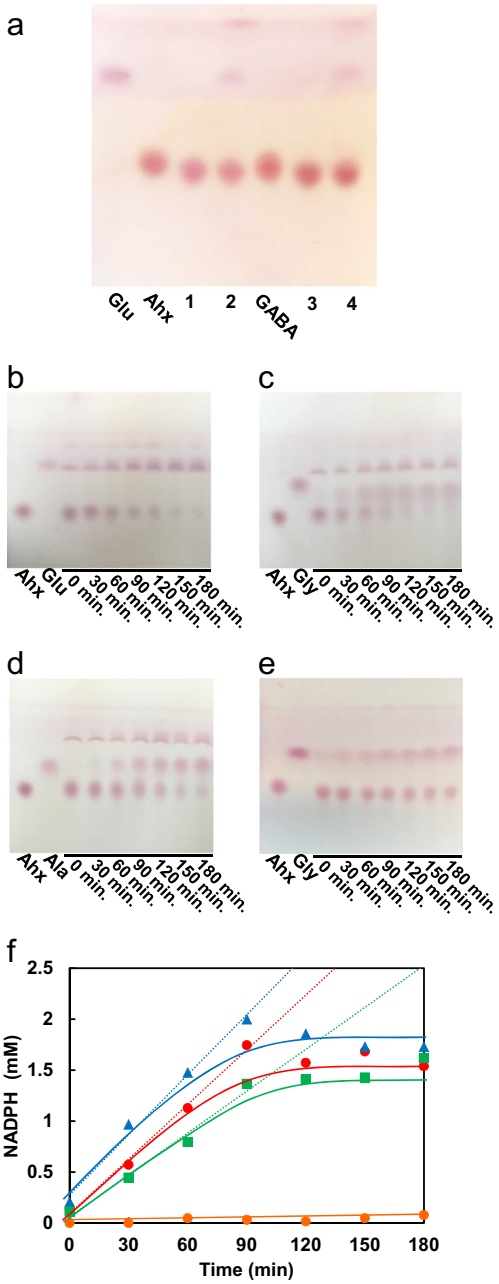

Fig. 5 Detection of $NylD_1$ activity. **a** Reactions were performed using 5 mM amino donor (Ahx or GABA), 5 mM α-KG, 0.1 mM PLP, and $NylD_1$ (0.15 mg/ml) in 100 mM KPB (pH 7.8) at 30 °C for 16 h, and the reaction products were detected by TLC. Slot 1, reaction from Ahx without $NylD_1$; slot 2, reaction from Ahx; slot 3, reaction from GABA without $NylD_1$; slot 4, reaction from GABA. **b**–**e** Time course of the $NylD_1$/$NylE_1$ coupled reaction. Purified $NylD_1$ (0.1 mg/ml) and $NylE_1$ (0.1 mg/ml) were incubated at 30 °C with 2 mM Ahx (amino donor) and 2.5 mM of the amino acceptor [α-KG (**b**), glyoxylate (**c**), and pyruvate (**d**)], 0.1 mM PLP, 2.5 mM $NADP^+$, 100 mM KPB (pH 7.8). The reaction mixtures were sampled at 30-min intervals (up to 180 min), and the reaction products were analyzed by TLC. As a control experiment, transamination from Ahx to glyoxylate was investigated in the absence of $NylE_1$ and $NADP^+$ (**e**). Authentic Ahx, GABA, L-Glu, Gly, and L-Ala were spotted on TLC plates. **f** To quantify the rate of NADPH formation, the aliquots were suitably diluted, and the A_{340} was measured. α-KG (*red circles*), glyoxylate (*blue triangles*), pyruvate (*green squares*). Time course of the reaction using glyoxylate in the absence of $NylE_1$ and $NADP^+$ was shown as *orange circles*. The initial reaction rate was estimated from the dashed line for each substrate (Table 2).

$NylE_1$ We assayed the dehydrogenase activity of $NylE_1$ using chemically synthesized ASA or SSA as the substrate and $NADP^+$ as a coenzyme (Fig. 6). We estimated the initial reaction rate by monitoring the absorbance of NADPH at 340 nm (A_{340}). After the reaction was started, NADPH was formed from either ASA or SSA. However, the specific activity of ASA (18 μmol/min (U)/mg-protein) is approximately 500-fold of the activity of SSA (0.034 U/mg-protein). We confirmed that the initial reaction rate is proportional to the enzyme concentration up to 0.0016 mg/ml (for ASA) and

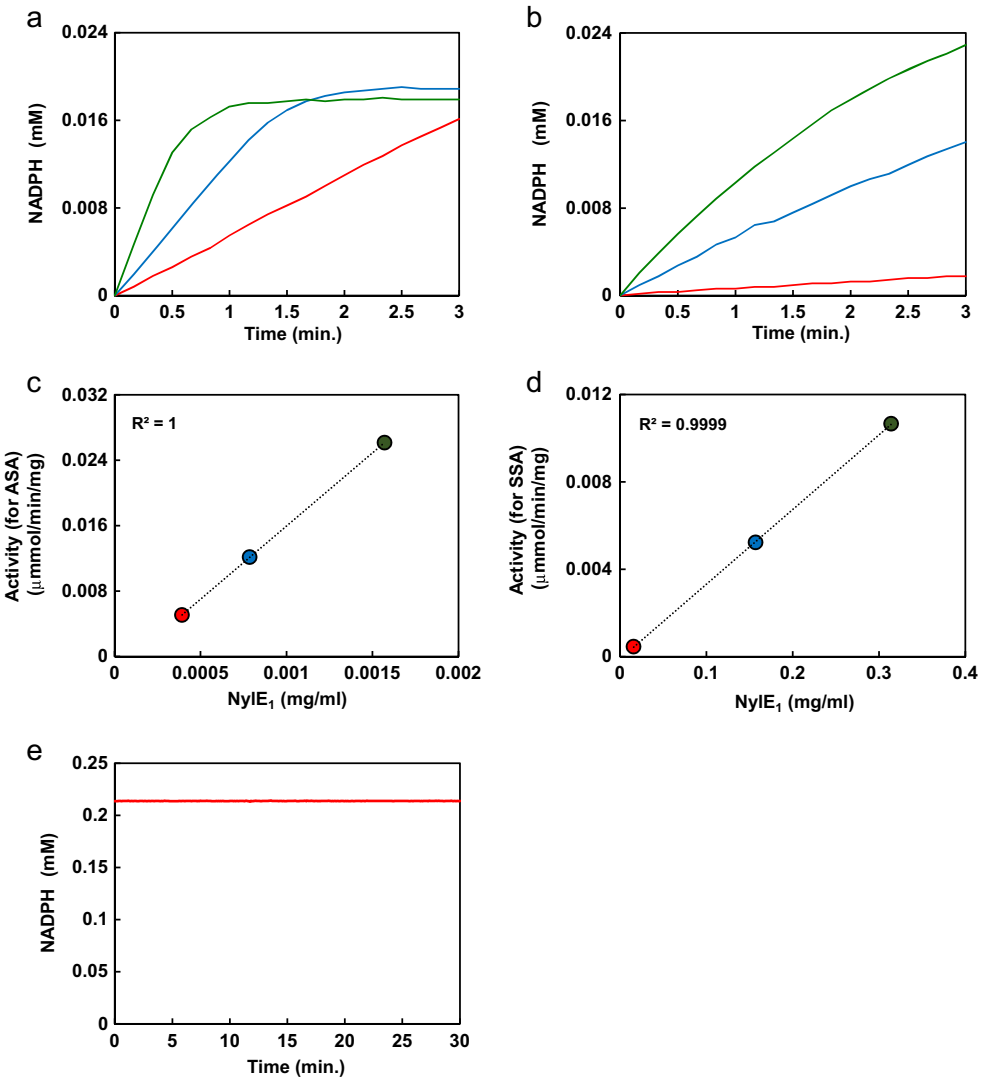

Fig. 6 Time course of $NylE_1$ reaction. **a** For the assay of the oxidative activity, $NylE_1$ (0.0004, 0.008, and 0.0016 mg/ml) was incubated at 30 °C with 0.5 mM ASA and 0.1 mM $NADP^+$ in 100 mM PPB (pH 7.8). The progress of the reactions was monitored by the increase in the A_{340}. **b** Enzyme reactions were similarly performed using 0.5 mM SSA and $NylE_1$ (0.016, 0.16, and 0.31 mg/ml). **c**, **d** Initial reaction rates for ASA (**c**) and SSA (**d**) were plotted for the protein concentrations of $NylE_1$. **e** For the assay of the reverse reaction of $NylE_1$, enzyme reactions were performed using $NylE_1$ (0.05 mg/ml), 0.25 mM NADPH, and 0.2 mM adipate in 100 mM PPB (pH 7.8), and the decrease in the A_{340} was monitored

0.3 mg/ml (for SSA) (Fig. 6c, d). These results demonstrate that $NylE_1$ has a much higher specific activity with the substrate responsible for Ahx/adipate metabolism than for GABA/succinate metabolism. However, it should be noted that the reaction apparently stops when approximately 20% of the $NADP^+$ is converted to NADPH (Fig. 6a). We estimate that the net concentrations of ASA or SSA involved in the reaction seemed to be decreased by spontaneous oxidation and/or polymerization reactions in aqueous solution due to the instability of the semialdehyde. However, as stated below, the coupled reaction using $NylD_1$/$NylE_1$ almost completely converts the Ahx to adipate. Therefore, we have concluded that ASA formed by the reaction with $NylD_1$ is successively converted to adipate by the reaction with $NylE_1$.

To test whether $NylE_1$ catalyzes the reductive reaction from adipate to ASA, we performed the reactions using purified $NylE_1$ (0.05 mg/ml: 31-fold concentrated enzyme used for the oxidative reaction), 0.2 mM adipate, and 0.25 mM NADPH in KPB buffer (pH 7.8). However, we found no detectable conversion of NADPH to $NADP^+$ by A_{340} even after the reaction ran for 30 min (Fig. 6e). On the basis of these findings, we have concluded that $NylE_1$ is highly specific for the oxidative activity of ASA and uses $NADP^+$ as a cofactor.

Construction of $NylD_1$/$NylE_1$ coupled reaction system

To establish a quantitative assay system for Ahx aminotransferase activity, we investigated the applicability of a $NylD_1$/$NylE_1$ coupled system using the purified enzymes ($NylD_1$, $NylE_1$), substrates (amino-donor and amino-acceptor), and coenzymes ($NADP^+$ and PLP) (Fig. 7a). As stated below, we demonstrate that the reaction system has great advantages for monitoring the aminotransferase activity.

We confirmed that $NylD_1$ has aminotransferase activity either for Ahx or GABA using α-KG as an amino acceptor (Fig. 5a). Especially in the coupled reaction where the reaction

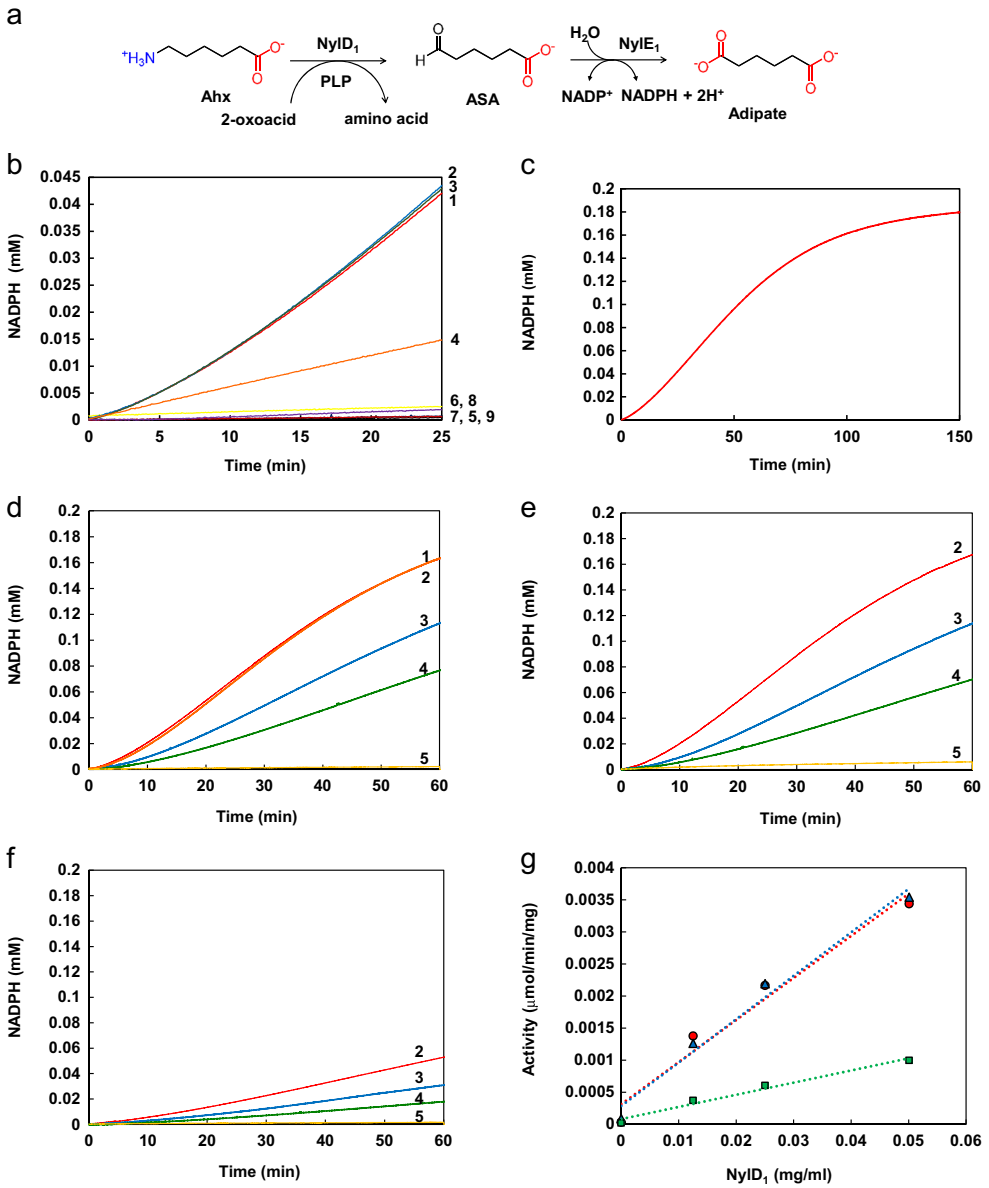

Fig. 7 Time course of $NylD_1/NylE_1$ coupled reaction. **a** A reaction scheme showing the conversion of Ahx to adipate by the sequential reactions of $NylD_1$ and $NylE_1$. **b** Factors responsible for the enzyme activity were tested by the coupled reaction using $NylD_1$ (0.05 mg/ml) and $NylE_1$ (0.1 mg/ml). The enzymes were incubated at 30 °C with 0.2 mM amino donor (Ahx), 0.25 mM of the amino acceptor (2-oxo acid), 0.1 mM PLP, and 0.25 mM $NADP^+$ in 100 mM KPB (pH 7.8) (standard assay condition) including 1 mM EDTA and 1 mM DTT. The conversion to NADPH was continuously quantified from A_{340}. 1, reaction mixture containing all components. 2–9, reaction mixture omitting the following components: DTT (2), EDTA (3), PLP (4), $NylD_1$ (5), $NylE_1$ (6), Ahx (7), αKG (8), $NADP^+$ (9). Initial reaction rates are summarized in Table 1. **c** The reaction (containing all components in **b**) was continued up to 150 min, and the time course of the formation of NADPH is shown. **d**–**f** Time course of $NylD_1/NylE_1$ coupled reaction system at different enzyme concentrations. The coupled reaction was performed by changing the ratio of $NylD_1$ and $NylE_1$ activity in the standard assay conditions. Enzyme activity was assayed using α-KG (**d**), glyoxylate (**e**), or pyruvate (**f**) as amino acceptor. $NylD_1/NylE_1 = 0.05/0.1$ mg/ml (1); 0.05/0.05 mg/ml (2); 0.025/0.05 mg/ml (3); 0.0125/0.05 mg/ml (4); 0/0.05 mg/ml (5). **g** Aminotransferase activity was calculated by subtracting the background level ($[NylD_1] = 0$, line 5) from the reaction rate in the complete system. The activity was plotted for the protein concentrations of $NylD_1$. α-KG (*red circles*); glyoxylate (*blue triangles*); pyruvate (*green squares*)

rate of $NylE_1$ is much greater than that of $NylD_1$, Ahx should be immediately converted to adipate without the accumulation of ASA. Therefore, the system makes it possible to monitor the activity continuously via the A_{340}. Since the high specific activity of $NylE_1$ for ASA is suitable for quantification of the Ahx aminotransferase activity, we analyzed the activity of the coupled system. The reaction proceeds at constant rate up to 55% conversion (approximately 0.11 mM NADPH at 60 min) in a reaction mixture containing all of the essential components (Fig. 7c). Moreover, even if the reaction is started by

using a two-fold greater amount of $NylE_1$ enzyme, the reaction rate was barely affected (Fig. 7d, curves 1 and 2). For the reaction using $NylD_1/NylE_1$ = 0.05/0.05 mg/ml (Fig. 7d curve 2), the $NylE_1$ activity for the synthetic ASA is calculated to be 0.9 U/ml from a specific activity of 18 U/mg (Fig. 6). However, the observed activity in $NylD_1/NylE_1$ coupled reaction is approximately 0.0033 U/ml (specific activity = 0.066 U/mg, Tables 1 and 2), indicating that the rate-limiting step in the sequential reaction is the $NylD_1$ reaction. Therefore, we have concluded that aminotransferase reaction occurs in the presence of a great excess of the semialdehyde dehydrogenase (NylE) activity.

In a reaction system where either $NylD_1$, $NylE_1$, Ahx, α-KG, or $NADP^+$ was absent, the activity drastically decreased to a background level (approximately 1.2–3.8% of the activity of the complete system; Table 1, Fig. 7b). However, even in a reaction mixture lacking PLP, approximately 26% of the activity is retained. The implication of this result is discussed below. Additionally, we found that DTT or EDTA has no detectable effect on the reaction rate (Table 1), although some bacterial semialdehyde dehydrogenases are stabilized and/or activated by DTT (Sanchez et al. 1989) but inhibited by the metal chelating agent EDTA (de Carvalho et al. 2011).

To confirm the acceptor specificity in the aminotransferase reaction, we tested the activity of $NylD_1$ using pyruvate or glyoxylate as the amino acceptor instead of α-KG (Fig. 7e, f). We found that $NylD_1$ utilizes αKG and glyoxylate as the amino acceptor at almost similar levels, whereas the activity of pyruvate was found to be 30% (assay 1) to 70% (assay 2) of the level of αKG (Table 2). A TLC analysis of the reaction mixtures revealed that the amino acids glutamate, alanine, and glycine are produced from Ahx and oxo-acids during the course of the reaction (Fig. 5). With αKG or glyoxylate as the amino acceptor, approximately 50% of the conversion was achieved after the reaction runs for 60 min, and Ahx is almost completely converted to glutamate and glycine after the reaction runs for 180 min (Fig. 5b, c). In a control experiment using glyoxylate and $NylD_1$ without $NylE_1$, approximately 50% of the conversion was achieved after 180 min (Fig. 5e). Thus, $NylD_1$ utilizes the three oxo-acids as amino acceptors, although the activity was found to be in the order of glyoxylate, α-KG, and pyruvate (Table 2). We have concluded from these findings that the lower level of conversion caused by the reversible reaction of $NylD_1$ is enhanced by coupling it to the $NylE_1$ reaction.

Table 1 6-Aminohexanoate aminotransferase activity by $NylD_1/NylE_1$ coupled system. Factors responsible for the catalytic activity

Composition	Specific activity (μmol/min/mg)	Relative activity (%)
All components	4.2×10^{-2}	100
- DTT	4.4×10^{-2}	105
- EDTA	4.3×10^{-2}	102
- PLP	1.1×10^{-2}	26
- $NylD_1$	5.0×10^{-4}	1.2
- $NylE_1$	1.6×10^{-3}	3.8
- Ahx	7.1×10^{-4}	1.7
- αKG	1.1×10^{-3}	2.6
- $NADP^+$	4.5×10^{-4}	1.1

Enzyme reactions were performed under the standard assay conditions for a NylD/NylE coupled reaction system (0.2 mM Ahx, 0.25 mM α-KG, 0.1 mM PLP, and 0.25 mM $NADP^+$ in 100 mM KPB, pH 7.8) containing 1 mM DTT and 1 mM EDTA as a common component. Details are described in the "Materials and methods" section. In Tables 1 and 2, enzyme samples obtained by independent purification experiments were used

Table 2 6-Aminohexanoate aminotransferase activity by $NylD_1/NylE_1$ coupled system. Specificity of amino acceptor

Amino acceptor	Specific activity (μmol/min/mg)	
	Assay 1	Assay 2
α-KG	0.066	0.16
Glyoxylate	0.068	0.21
Pyruvate	0.019	0.11

Enzyme activities were quantified by two assays, which differed in the concentrations of amino donor (Ahx), amino acceptor (α-KG, pyruvate, glyoxylate), and $NADP^+$. Assay 1, 0.2 mM Ahx; 0.25 mM of the amino acceptor; 0.25 mM $NADP^+$. Assay 2, 2 mM Ahx; 2.5 mM of the amino acceptor; 2.5 mM $NADP^+$. In Tables 1 and 2, enzyme samples obtained by independent purification experiments were used

Discussion

We have confirmed that 6-nylon monomer, 6-aminohexanoate (Ahx), is converted to adipate by sequential reactions of Ahx aminotransferase ($NylD_1$) and ASA dehydrogenase ($NylE_1$) in *Arthrobacter* sp. strain KI72. An enzyme assay using a $NylD_1/NylE_1$ coupled system revealed that $NylD_1$ requires PLP as a cofactor for the aminotransferase activity. Generally, PLP-dependent enzymes are involved in the biosynthesis of amino acids and amino acid-derived metabolites, in which PLP acts as a coenzyme for the transamination, deamination, racemization, and decarboxylation reactions (Eliot and Kirsch 2004; Percudani and Peracchi 2003; Hwang et al. 2005; Phillips 2015; Steffen-Munsberg et al. 2015; Schiroli and Peracchi 2015).

GABA transferases from pig (Pig-GABT) (Storici et al. 2004), *E. coli* (Eco-GABT) (Liu et al. 2004), and *Arthrobacter aurescens* (Ara-GABT) (Bruce et al. 2012) have been structurally and biochemically characterized. The ε-amino group of the active site Lys generally forms a Schiff base with the aldehydic carbon of PLP (Eliot and Kirsch 2004)

at the catalytic center of the PLP-dependent enzymes. Since approximately 26% of the activity is retained if the PLP is left out of the reaction mix (Table 1), we estimate that a portion of PLP is bound to the enzyme even after purification has been carried out. An X-ray crystallographic analysis of Ara-GABT aminotransferase complexed with PLP has revealed that the ε-amino group of Lys295 formed a Schiff base (Bruce et al. 2012). Lys268 in the *E. coli* enzyme (Eco-GABT) is identified to be connected to PLP (Liu et al. 2004). From the alignment of the amino acid sequences, the active site Lys295 in Ara-GABT and Lys268 in Eco-GABT is conserved as Lys295 in $NylD_1$ (Fig. 2b). Thus, the conserved PLP-binding site and presence of Ahx- and GABA-aminotransferase activity of $NylD_1$ demonstrate that the unnatural amino acid Ahx is metabolized as an analog of physiological substrate GABA via a similar catalytic mechanism as reported for most PLP-dependent aminotransferases.

Of the 20 putative aldehyde dehydrogenases ($NylE_1$–$NylE_{20}$) identified in *Arthrobacter* strain KI72, $NylE_1$ had the highest homology (63.5%) with adipate semialdehyde (6-oxohexanoate) dehydrogenase (i.e., the *chnE* gene from *Rhodococcus*) (Fig. 3; Iwaki et al. 1999). Actually, since the specific activity of $NylE_1$ for ASA (18 U/mg) is approximately 500-fold of the activity of SSA (0.034 U/mg) (Fig. 6), we think that $NylE_1$ is specific for Ahx/adipate metabolism rather than GABA/succinate metabolism, as stated above.

The *gabT* (GABA amino transferase) and *gabD* (succinate semialdehyde dehydrogenase) genes responsible for GABA metabolism constitutes an operon in the *E. coli* chromosome (Bartsch et al. 1990). In contrast, the $nylD_1$ and $nylE_1$ genes are located on the contig sequences no. 23 and 1, respectively. This result indicates that $nylD_1$ and $nylE_1$ genes are not linked in the genomic DNA of strain KI72 (Figs. 2 and 3). It should be noted that $nylD_2$, and most of the $nylE_2$–$nylE_{20}$ genes are distributed at various loci on the chromosomal DNA of strain KI72. In contrast, the *nylBC* genes responsible for the hydrolysis of Ahx oligomers and nylon-6 are located on a plasmid in *Arthrobacter*, but these genes are chromosomal in *Agromyces* and *Kocuria* (Yasuhira et al. 2007a). Moreover, we have suggested that at least two genetic recombinations, i.e., the IS*6100*-mediated transposition of *nylB/nylC* gene region and a recombination that generates hybrid gene structures for the analogous *nylB'*/*nylC'* regions, are involved in the genetic organization in the alkalophilic nylon-oligomer degradative bacterium *Agromyces* (Yasuhira et al. 2007b). In addition, a plasmid-encoded NylA from *Arthrobacter* and *Pseudomonas* and ω-laurolactam hydrolase from *Cupriavidus*, *Rhodococcus*, and *Sphingomonas* are found to have 98–99% overall homology, although these strains have been classified into different genera (Yasuhira et al. 2010). These results may imply that bacterial strains have evolved a novel metabolic pathway for nylon-6-related compounds by assembling the responsible genes into the parental genomic DNA.

We have found that 6-aminohexanoate oligomers are almost completely converted to the monomers (Ahx) by the three nylon oligomer-degrading enzymes, NylA, NylB, and NylC (Negoro 2000, Negoro et al. 2005, 2007, 2012; Nagai et al. 2014). In addition, the present study has revealed that the yield for the conversion of Ahx to adipate is estimated to be > 90% on the basis of the stoichiometry of the overall reaction in the coupled system (Figs. 5 and 7). On the basis of these findings, we demonstrate that bio-based conversion from wastes/byproducts of nylon-6 to adipate (nylon-66 monomer) is possible, when the NADPH generated by the NylE reaction is re-oxidized to $NADP^+$ by coupling a suitable oxidoreductase reaction and the enzyme activity is high enough at a practical level.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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