

ϵ -Poly-L-lysine dispersity is controlled by a highly unusual nonribosomal peptide synthetase

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ϵ -Poly-L-lysine (ϵ -PL) consists of 25–35 L-lysine residues in isopeptide linkages and is one of only two amino acid homopolymers known in nature. Elucidating the biosynthetic mechanism of ϵ -PL should open new avenues for creating novel classes of biopolymers. Here we report the purification of an ϵ -PL synthetase (Pls; 130 kDa) and the cloning of its gene from an ϵ -PL-producing strain of *Streptomyces albulus*. Pls was found to be a membrane protein with adenylation and thiolation domains characteristic of the nonribosomal peptide synthetases (NRPSs). It had no traditional condensation or thioesterase domain; instead, it had six transmembrane domains surrounding three tandem soluble domains. These tandem domains iteratively catalyzed L-lysine polymerization using free L-lysine polymer (or monomer in the initial reaction) as acceptor and Pls-bound L-lysine as donor, directly yielding chains of diverse length. Thus, Pls is a new single-module NRPS having an amino acid ligase-like catalytic activity for peptide bond formation.

Two amino acid “homopolymers” comprising a single type of amino acid are known in nature¹: γ -polyglutamic acid (γ -PGA) and ϵ -PL. The latter, which consists of 25–35 L-lysine (**1**) residues with linkages between α -carboxyl groups and ϵ -amino groups (Fig. 1a), has antimicrobial activity against a spectrum of microorganisms including bacteria and fungi¹. Owing to its safety and biodegradability, it is used as a food preservative in several countries. The biological activity of ϵ -PL is dependent on its molecular size. The relationship between the molecular size of ϵ -PL and its antimicrobial activity against *Escherichia coli* K-12 has been investigated (ref. 2). ϵ -PL with more than nine L-lysine residues severely inhibits microbial growth; however, the L-lysine octamer demonstrates negligible antimicrobial activity. In contrast, chemically synthesized α -poly-L-lysine containing a considerably longer chain of L-lysine residues (50 residues), which show linkages between the α -carboxyl and α -amino groups, demonstrates a lower activity than ϵ -PL. Thus, polymerization of L-lysine via an isopeptide bond is required in order for ϵ -PL to exert its biological activity, and the polymerization mechanisms involved in the chain-length diversity of ϵ -PL are of particular interest.

NRPSs are multifunctional enzymes consisting of semiautonomous domains that synthesize numerous secondary metabolites^{3–6}. Using an assembly-line logic comprising multiple modules, they use a thiotemplated mechanism to activate, tether and modify amino acid monomers, sequentially elongating the peptide chain before releasing the complete peptide. The order and number of modules of an NRPS system determine the sequence and length of the peptide product. It has been reported that ϵ -PL might be produced by NRPSs using a thiotemplated mechanism^{7,8}; however, as the investigators used the

crude extract of an ϵ -PL-producing microorganism, it was not possible to confirm the biosynthetic mechanism. Additionally, the chain-length diversity of ϵ -PL products is difficult to explain with this generic model. We recently identified and characterized ϵ -PL-degrading enzymes in the ϵ -PL producer *S. albulus* NBRC14147 (ref. 9). This raised the question of whether the degrading enzymes, rather than the biosynthetic machinery, might be responsible for generating products of diverse chain length. This report describes the purification and characterization of an ϵ -PL synthetase (Pls) to determine its polymerization mechanism and investigate how chain-length diversity is generated. We also demonstrate the ability of the Pls to accommodate substrate diversity, which will facilitate further elucidation of the biosynthetic mechanism of ϵ -PL and serve as a first step in the development of new classes of biopolymers.

RESULTS

Purification of Pls from *S. albulus* NBRC14147

S. albulus NBRC14147, which is an ϵ -PL producer, was grown to the late logarithmic phase, in which ϵ -PL production was observed. The mycelia collected from a 700-ml culture broth were sonicated and centrifuged to obtain a cell-free extract. After ultracentrifugation of the cell-free extract, ϵ -PL synthesis was observed in an insoluble fraction, which suggests that Pls is an insoluble protein such as a membrane protein. We therefore solubilized this fraction with the non-ionic detergent nonidet P-40 (NP-40). By successive purification steps including column chromatography, the solubilized Pls was finally purified 168-fold to apparent homogeneity (see Methods and **Supplementary Table 1** online). The relative molecular mass of the native

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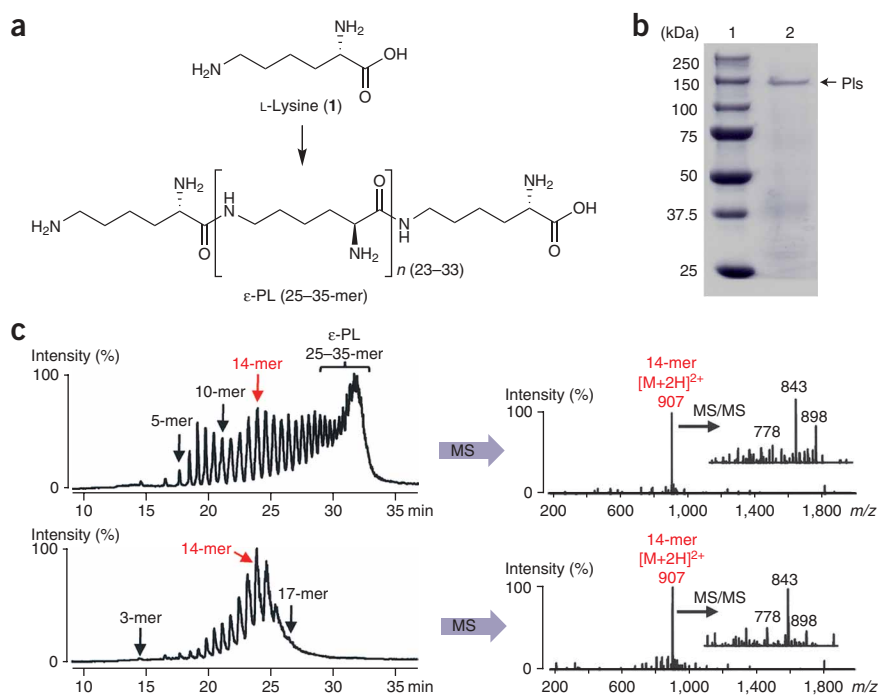


Figure 1 *In vitro* polymerization of L-lysine (1) with Pls purified from *S. albulus* NBRC14147. (a) Chemical structures of L-lysine and ε-PL (25-mer to 35-mer). (b) Pls purified to homogeneity (lane 2) was analyzed by SDS-PAGE together with a molecular mass standard (lane 1). Proteins were stained with Coomassie Brilliant Blue (CBB) R-250. (c) Identification of the Pls reaction products. The reaction mixture (lower chromatogram and ESI mass spectrum) and a hydrolysate of ε-PL (upper chromatogram and ESI mass spectrum) were analyzed by HPLC/ESI-MS as described in the Methods. The ε-PL hydrolysates (0.1 mg ml⁻¹) were prepared by hydrolysis with 1N HCl.

enzyme as estimated by gel filtration chromatography was 270 kDa (Supplementary Fig. 1a online). However, the molecular mass as estimated by denaturing SDS-PAGE was 130 kDa (Fig. 1b), which suggests that Pls is a homodimer.

Enzymatic characterization of the purified Pls

To confirm that the purified enzyme is a Pls, the protein was incubated with L-lysine and ATP, and the reaction mixture was analyzed by HPLC/ESI-MS. A polydisperse group of enzyme-dependent polymer products were detected, with their retention times corresponding to those of reference standard polymers consisting of 3–17 residues (Fig. 1c). Incubation of Pls with L-lysine for different times did not change the relative amounts of each polymer produced during the polymerization reaction (Supplementary Fig. 1b). ESI-MS/MS analysis of the enzymatically synthesized polymer with 14 residues was identical to that of the reference standard polymer with 14 residues (Fig. 1c); mass spectra identical to the reference standards were also observed for products of other chain lengths (Supplementary Table 2 online). Chemical modification of the synthesized polymers using 2,4-dinitrophenol (2, DNP) followed by TLC analysis against reference DNP-modified α-lysine and ε-lysine polymers demonstrated that the α-amino groups of the L-lysine residues were labeled with DNP (Supplementary Fig. 1c). This result shows that the polymer consists of L-lysine residues with linkages between the α-carboxyl and ε-amino groups, and it confirms that the polydisperse polymer products are indeed ε-PL.

Other enzymatic properties of Pls were further investigated. The enzyme required Mg²⁺, ATP, 20–30% glycerol (v/v), 2 mM dithiothreitol and 0.2–0.4% NP-40 (v/v) for full activity. ATP was converted to AMP during the Pls reaction (Supplementary Fig. 1d). No activity was detected with other nucleotides such as GTP, CTP and TTP in the Pls polymerization reaction (Supplementary Fig. 1e). Maximum activity occurred at an optimum pH of 8.5 (Supplementary Fig. 1f). The effect of temperature on enzyme activity was investigated over the range of 10–45 °C, and the maximum activity was observed at 25–30 °C (Supplementary Fig. 1g).

Cloning, sequence analysis and inactivation of Pls

To clone the gene encoding Pls, we first needed to determine some of the amino acid sequence of Pls. Tryptic digests of Pls were analyzed by HPLC/ESI-MS, and two internal amino acid sequences, N-A(I/L)AGT(I/L)KPGAYPR-C and N-MAGAAWPAWQRSR-C, were obtained. Based on these sequences, we designed PCR primers. Using the PCR product as a probe, we cloned a 33-kilobase-pair DNA fragment containing the *pls* gene from *S. albulus* NBRC14147 (Supplementary Fig. 2a online). Notably, a BLAST database search showed that homologous genes are widely distributed among microorganisms (Supplementary Table 3 online). The *pls* gene encodes a protein of 1,319 amino acids containing the internal amino acid sequences previously determined. The calculated molecular mass (138,385 Da) was in good agreement with the result from the SDS-PAGE analysis of the purified Pls (Fig. 1b). Additionally, a knockout mutant of the putative *pls* gene produced no ε-PL (Supplementary Fig. 2b), which provides strong support that we had identified Pls.

With the protein sequence in hand, we determined the predicted function of the enzyme. In a traditional NRPS, the amino acid substrate is activated as an acyl-O-AMP by an adenylation domain (A domain)^{3–6} and subsequently loaded onto the 4'-phosphopantetheine (4'-PP) arm of the adjacent thiolation domain (T domain)^{3–6}, thereby resulting in the formation of an acylthioester and AMP release. A domain search showed the presence of an A domain and a T domain in the N-terminal region of the Pls. Our previous observation that AMP is released during the course of the reaction—along with (i) the similarity of the ten-residue sequence that confers substrate specificity in the putative Pls A domain to that of the

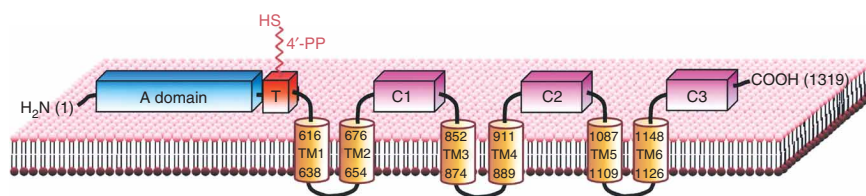


Figure 2 Domain architecture of Pls. The A domain, T domain, six TM domains and three tandem domains (C1 domain, C2 domain and C3 domain) are shown schematically. The numbers on Pls are the amino acid residue numbers.

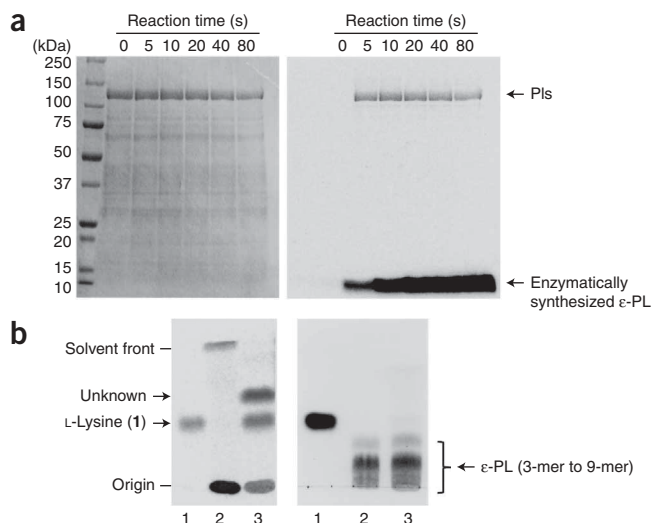


Figure 3 Performic acid oxidation of the radiolabeled Pls formed in the Pls reaction with L-[U- ^{14}C]lysine. **(a)** After incubation of Pls with L-[U- ^{14}C]lysine and ATP at 5 °C for 0–80 s, the reaction mixtures were subjected to SDS-PAGE (5–20% gradient gel, 16 μg protein per lane). Proteins were stained with CBB R-250 (image on the left). The dried gel was exposed on an imaging plate and visualized by BAS-2500 (Fuji film; image on the right). **(b)** The reaction mixture incubated for 10 s was further used for the oxidation experiments of Pls. L-[U- ^{14}C]lysine (lane 1) and the radiolabeled Pls treated with performic acid (lane 3) and formic acid (lane 2) were analyzed by silica gel TLC (left image). The dried TLC was exposed on an imaging plate and visualized by BAS-2500. Additionally, to confirm that these acids do not hydrolyze the short-chain ϵ -PL oligomers, the oligomers (3-mer to 9-mer) were treated with formic acid (lane 2) and performic acid (lane 3) and analyzed by silica gel TLC (image on the right). L-Lysine (lane 1) was also used as the reference standard to confirm the mobility of the short-chain ϵ -PL oligomers. The samples were detected with ninhydrin reagent. In these oxidation experiments, an amino acid bound to the enzyme as a thioester is released from the enzyme not by formic acid but by performic acid. In the left image of **b**, we detected an extra band (lane 3), which is known as the commonly observed unknown spots in performic acid oxidation experiments¹⁹.

A domain of the BacB (ref. 10) protein that adenylates L-lysine (Supplementary Table 4 online), and (ii) the fact that Ser553 from the putative T domain is in proper alignment to bind 4'-PP_i in contrast to the T domains of traditional NRPSs (Supplementary Table 5 online)—provides strong support for the classification of these regions of Pls as A domains and T domains. This distinguishes the mechanism of ϵ -PL biosynthesis from that of γ -PGA (ref. 1), glutathione (3)¹¹ and cyanophycin¹, which require phosphorylation of the carboxyl group.

Surprisingly, given the evidence for the presence of the A domains and T domains, Pls had no domain with significant sequence similarity to the traditional condensation domains (C domains) that are crucial in peptide bond formation in NRPSs^{3–6}. Furthermore, it had no traditional thioesterase domain (TE domain), which catalyzes release of the final product from NRPS enzymes by hydrolysis to the free acid or cyclization to an amide or ester^{3–6}. Instead, a physicochemical analysis of the Pls amino acid sequence with SOSUI (ref. 12) suggested the existence of six transmembrane domains (TM domains)

surrounding three tandem soluble domains that display substantial sequence similarity (with pairwise identities of 27%, 22% and 23%; Supplementary Fig. 2c) (Fig. 2). Alignment of the tandem domains with traditional C domains demonstrated that the tandem domains do contain motifs showing similarity to histidine motifs (the HHxxxDG sequences found in all traditional C domains), but the two histidine residues, which are critical for catalysis, are not conserved (Supplementary Fig. 2d). However, both the primary sequence and the predicted three-dimensional structure of these domains showed similarity to acetyltransferases (Supplementary Fig. 2e), which do show structural similarity to C domains^{13,14}. For this reason, we named the tandem sequences the C1, C2 and C3 domains, with the expectation that they would have a role in peptide bond formation.

Investigation of the Pls mechanism

We next explored the catalytic mechanism of Pls using an ATP-PP_i exchange assay. We observed Pls-mediated adenylation of L-lysine, but not of any other proteinogenic amino acid (Supplementary Fig. 3a

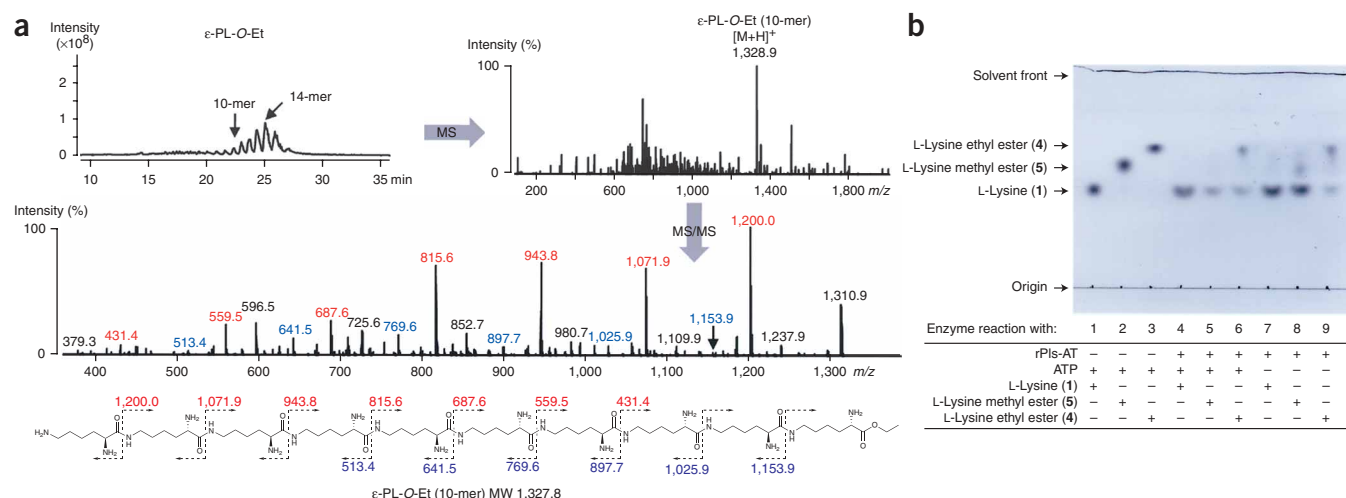
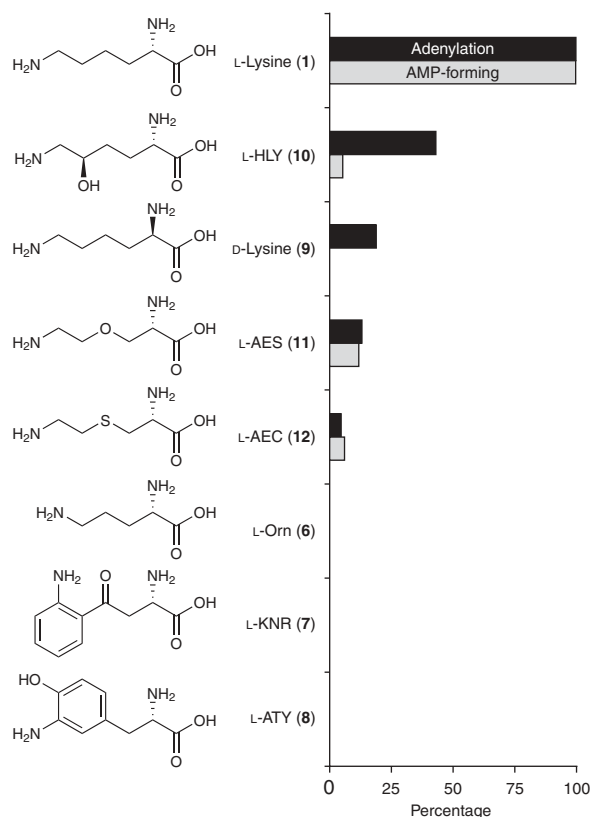


Figure 4 Pls reactions with L-lysine esters. **(a)** The Pls polymerization reaction with 2 mM L-lysine ethyl ester (4) was performed and analyzed by HPLC/ESI-MS. The mass spectra and MS/MS spectra of the selected polymers (10-mer) in the reactions are shown. The reaction conditions are described in the Methods. **(b)** The rPls-AT reaction with 2 mM L-lysine (1), 2 mM L-lysine methyl ester (5) or 2 mM L-lysine ethyl ester (4) was performed and analyzed by silica gel TLC. TLC was developed in 1-butanol/pyridine/acetic acid/H₂O, 2:1:1:2 (v/v). The products were detected with ninhydrin reagent. These results demonstrate that the esterase activities were not dependent on ATP. Addition, (+); no addition, (-).



online), which confirmed our predicted substrate selectivity for lysine. To investigate the function of the putative T domain, we constructed a histidine-tagged recombinant Pls containing only the A domain and T domain (rPls-AT) using *Streptomyces lividans* TK23 as a heterologous host. Incubation of rPls-AT with L-[U-¹⁴C]lysine and ATP as substrates resulted in loading of the labeled lysine onto the enzyme (Supplementary Fig. 3b). However, ε-PL production was not detected, which suggests that the three tandem domains (C1 domain, C2 domain and C3 domain) of Pls are essential for catalyzing the

Figure 5 Substrate specificities in the A domain and T domain of Pls. The relative ability of Pls to form AMP conjugates and to adenylate substrates was measured for L-lysine and several L-lysine analogues. Each value is represented as the mean of three experiments. Error bars are not shown because relative standard deviations of less than 5% were commonly calculated.

L-lysine polymerization reaction. To gain a better understanding of the function of the tandem domains, we constructed two additional recombinant Pls enzymes: rPls-ATC1C2 (lacking the C3 domain) and rPls-ATC1 (lacking both the C2 domain and C3 domain) (Supplementary Fig. 3c). As observed for rPls-AT, which lacks all three C domains, no polymer products were detected for rPls-ATC1C2 and rPls-ATC1, which suggests that either the C3 domain or the interconnected action of all three domains is essential for peptide bond formation.

As described earlier, Pls is predicted not to have a TE domain, which is traditionally required for release of the product from the NRPS machinery. This suggested that the growing polymer products are not covalently attached to Pls during the polymerization reaction. To investigate this hypothesis, Pls was incubated with L-[U-¹⁴C]lysine and ATP. In this experiment, we used low-temperature conditions (5 °C for 0–80 s) for the reaction, because we expected that by depressing the catalytic velocity, we would be able to observe more intermediates of the reaction. Analysis of the reaction mixture confirmed that Pls was radiolabeled during the reaction (Fig. 3a). However, performic acid treatment of the labeled Pls (which releases any small molecules attached via thioester bonds) returned only L-[U-¹⁴C]lysine monomers; no polylysine chains were observed (Fig. 3b). These results strongly suggested that the growing polymer products are not covalently attached to Pls during the polymerization reaction. We similarly observed that short-chain ε-PL oligomers (3-mer to 9-mer) are neither adenylated (data not shown) nor incorporated into polymers primed with free, deuterated L-lysine (L-Lys-d8); instead, Pls produced L-Lys-d8 homopolymers (Supplementary Fig. 3d). To further test this mechanism, we used L-lysine ethyl ester (4) and L-lysine methyl ester (5) as substrates. HPLC/ESI-MS analysis of the reaction revealed that Pls produced the

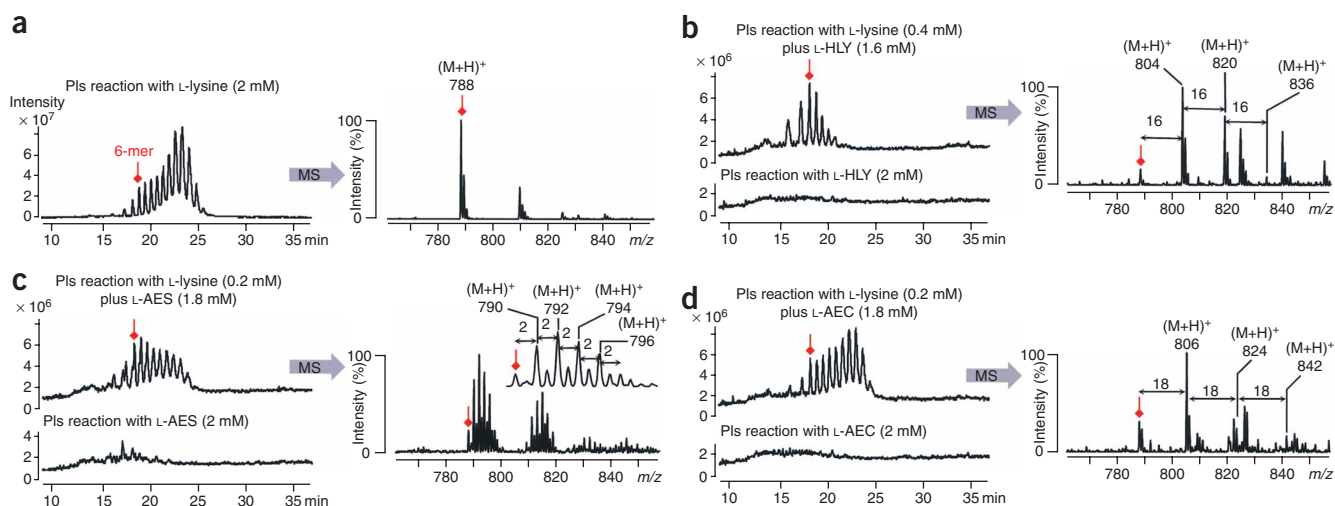


Figure 6 *In vitro* production of heteropolymers consisting of L-lysine and the L-lysine analogs. (a–d) The Pls reaction with 2 mM L-lysine (a), 1.6 mM L-HLY plus or minus 0.4 mM L-lysine (b), 1.8 mM L-AES plus or minus 0.2 mM L-lysine (c) or 1.8 mM L-AEC plus or minus 0.2 mM L-lysine (d) was performed and analyzed by HPLC/ESI-MS. The mass spectra of the selected polymers (6-mer) in the reactions are shown in the right-hand panels. The reaction conditions are described in the Methods.

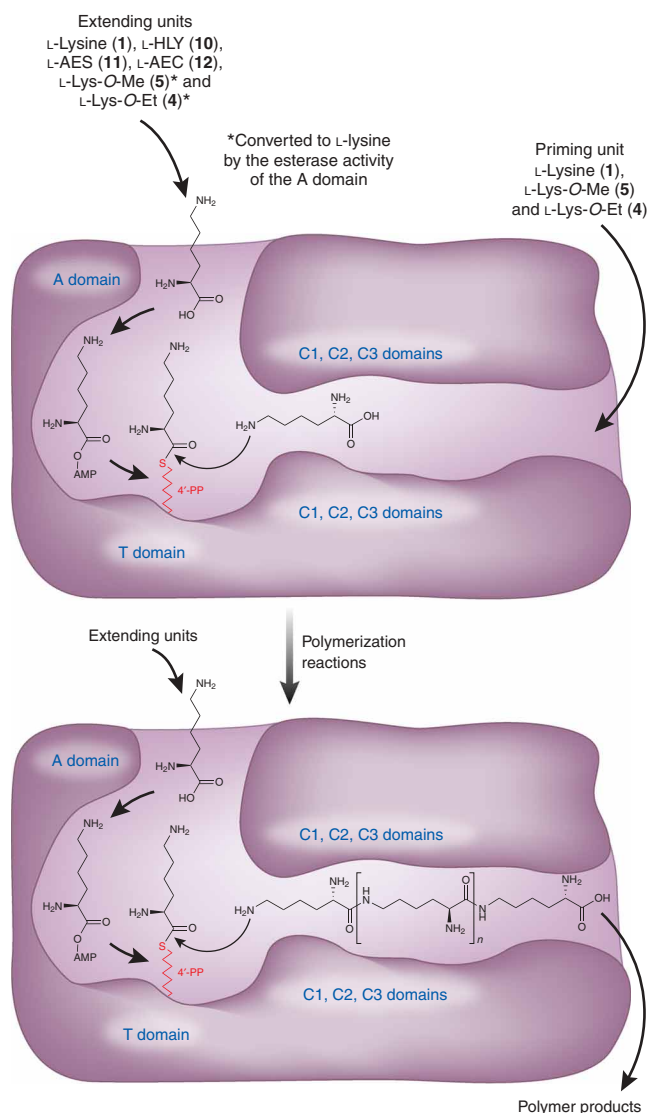


Figure 7 Pls polymerization mechanism and the proposed model of the Pls catalytic cavity with substrate specificity. The clarified polymerization mechanism is shown schematically. L-Lys-O-Me, L-lysine methyl ester; L-Lys-O-Et, L-lysine ethyl ester.

corresponding ethyl (**Fig. 4a**) and methyl ester forms of ϵ -PL (**Supplementary Fig. 3e**). Though retention of the final ester functionality confirmed that the growing polymers or polymer products are not tethered to Pls via covalent bonds such as thioesters or esters during the polymerization reaction (which would result in complete loss of the ester group), it also raised the question of how the enzyme catalyzes polymerization using ester substrates at all. The answer lay in a unique function of Pls: the A domain converts these L-lysine esters to L-lysine by its own esterase activity (**Fig. 4b**), and then the resulting L-lysine can be adenylated and loaded to the T domain.

Substrate specificity of Pls

Given that Pls can accept lysine esters as substrates, we wondered whether the protein would be tolerant of additional substrate diversity. The substrate specificity of Pls was therefore investigated further with seven L-lysine analogs (**Fig. 5**). L-Ornithine (**6**, L-Orn), L-kynurenine (**7**, L-KNR) and 3-amino-L-tyrosine (**8**, L-ATY) were not accepted as

substrates. Pls was able to adenylate D-lysine (**9**; relative activity = 19%), but AMP-forming activity was not detected, which suggests that the adenylated D-lysine is not loaded onto the T domain. Indeed, D-lysine serves as an inhibitor of the aminoacylation of L-[U- 14 C]lysine (**Supplementary Fig. 3b**), in agreement with a model where D-lysine is stalled on the A domain. Pls did successfully adenylate and pantetheinylate (presumed from observed AMP formation) the rest of the analogs, including (5R)-5-hydroxy-L-lysine (**10**, L-HLY; relative activity for adenylation, 43%; relative activity for AMP-formation, 6%), O-(2-aminoethyl)-L-serine (**11**, L-AES; corresponding values, 13% and 12%) and S-(2-aminoethyl)-L-cysteine (**12**, L-AEC; corresponding values, 5% and 6%). However, no homopolymers from these analogs were observed (see the lower chromatograms in **Fig. 6**). In contrast, when we added a small amount of L-lysine to the reaction mixtures, heteropolymers consisting of L-lysine and the analogs were produced (upper chromatograms in **Fig. 6**). These results indicate that the A domains and T domains are partially tolerant to substrate analogs, whereas the three tandem acetyltransferase domains show high specificity for L-lysine.

DISCUSSION

In this study, we successfully purified a membrane protein with six TM domains, from the membrane fraction of *S. albulus* NBRC14147, and characterized it as Pls, the biological machinery for ϵ -PL synthesis. Our explorations of Pls resulted in the development of a new model for the generation of chain-length diversity of ϵ -PL products that integrates traditional NRPS logic (in the form of A domains and T domains) with amino acid ligase functionality in three tandem domains that show similarity to both acetyltransferases and, through these transferases, traditional C domains. The catalytic mechanism is initiated in the N terminus by the A domains and T domains with the adenylation and transfer of an incoming L-lysine monomer (or 'extending unit'), with polymerization occurring as freely diffusible substrates (or 'priming units') are added by the C-terminal tandem domains to the extending unit (**Fig. 7**). Because this cycle has no predetermined endpoint, other than the loss of the noncovalently bound polymer chain to solution, Pls acts iteratively for ϵ -PL chain growth to obtain a multitude of chain lengths (observed in our studies as ranging from 3 to 17 residues). This mechanism, in which a single polymer is created and then released (**Fig. 7**), is supported not only by our characterization of more basic Pls properties such as ATP turnover and ϵ -amine linkage formation, but also by our observations that (i) there was no difference in the relative amounts of each polymer during the polymerization reaction, and (ii) preexisting short-chain polymers could not be incorporated into new chains (that is, the short chains were not simply intermediates of the longer chains).

Though ϵ -PL chain-length diversity can be explained by this mechanism, a detailed description of how the growing polymer interacts with the protein remains a subject for future work. We hypothesize that the protein contains a long slender-shaped tunnel, or cavity, that is continuously occupied by a growing polymer during the polymerization reaction. This would explain why the polymerization reaction is specific to the ϵ -amino groups of the priming units, as the similarly reactive α -amino groups could be buried or otherwise protected from reaction by the Pls catalytic cavity (**Fig. 7**).

In our analysis of the sequence of the three tandem domains, we identified a region that is reminiscent of the known histidine motif from traditional C domains, but that lacks the histidine residues thought to be required for catalysis. It has been reported recently that the two histidine residues in the histidine motif of VibH are also not critical for catalysis¹⁴. As VibH, the functional C domain of a

vibriobactin NRPS, uses a similar mechanism to Pls—catalyzing peptide bond formation between an NRPS-bound substrate, dihydroxybenzoate, and the freely diffusible substrate norspermidin—these combined results suggest that the absence or lack of necessity of the two histidine residues may define a secondary motif that can be used to identify C domains operating via characteristic amino acid ligase-like mechanisms.

Finally, our investigations demonstrated that the chain-length diversity of ϵ -PL is directly generated by the synthetase, rather than via the differential degradation of a uniform polymer by ϵ -PL-degrading enzymes. Future investigations may explore whether the activity of these degrading enzymes serves to create a shorter polymer with some defined function, or whether the enzymes simply regenerate the lysine building blocks from the unusual ϵ -PL chain architecture. Similarly, further explorations of the homologous genes identified in our BLAST search should prove useful, as their encoded proteins may synthesize amino acid homopolymers other than ϵ -PL and γ -PGA. Investigations of these proteins and further exploration of Pls should facilitate biosynthetic engineering and help to create new classes of biopolymers.

METHODS

Chemicals. L-[U- 14 C]lysine (11.2 GBq mmol $^{-1}$) was obtained from GE Healthcare, and [32 P]pyrophosphate (2.98 TBq mmol $^{-1}$) was obtained from Perkin Elmer Life Science. All other chemicals were of analytical grade. ϵ -PL (reference standard, **Supplementary Fig. 1h**) was obtained from Chisso Corporation.

Pls assay. The enzyme assay was based on an assay reported previously⁷. The reaction mixtures contained 100 mM *N*-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS)-NaOH (pH 8.5), 10 μ M L-[U- 14 C]lysine (112 kBq ml $^{-1}$), 990 μ M nonlabeled L-lysine, 5 mM MgCl $_2$, 5 mM ATP, 1 mM dithiothreitol (DTT), 20% glycerol (v/v), 0.2% (w/v) NP-40 and 4.0 μ l enzyme solution in a final volume of 40 μ l. The reaction mixtures were incubated at 30 °C for 10 min. All assays were carried out under linear conditions, and one unit was defined as the amount of enzyme catalyzing the incorporation of 1 pmol of L-lysine into ϵ -PL per second at 30 °C.

Purification of Pls. *S. albulus* NBRC14147 was grown in PLP medium⁹ at 30 °C to the late logarithmic phase (32 h cultivation in this work). The mycelia (75 g wet weight) from a 700-ml culture were harvested and sonicated in 150 ml buffer A (100 mM Tris-HCl, 20% glycerol (v/v), 2 mM EDTA, 0.2 M NaCl and 5 mM DTT (pH 8.0)). After centrifugation at 16,000g for 20 min, the supernatant was collected as a crude extract. This was centrifuged at 160,000g for 1 h, and the pellet was collected as the membrane fraction. This was then washed with buffer B (50 mM Tris-HCl, 20% glycerol (v/v), 1.0 M NaCl and 5 mM DTT (pH 8.0)) and used as the salt-washed membrane fraction. This fraction was sonicated in 30 ml buffer C (50 mM Tris-HCl, 30% glycerol (v/v), 1.0% NP-40 (v/v) and 5 mM DTT (pH 8.6)) and then ultracentrifuged again. The supernatant, the NP-40-solubilized fraction, was used for further purification. It was loaded onto a DEAE Toyopearl 650M column (2.5 \times 10 cm, Tosoh), pre-equilibrated with buffer D (50 mM Tris-HCl, 30% glycerol (v/v), 0.4% NP-40 (v/v) and 2 mM DTT (pH 8.6)) and eluted with a linear NaCl gradient (0–0.3 M) at a flow rate of 1.0 ml min $^{-1}$. Fractions with high enzyme activity were pooled, and the pH was adjusted to 8.2 with 0.1 M acetic acid. The pooled sample was loaded onto a triazine dye matrix AF-Blue Toyopearl 650M column (1.5 \times 6.5 cm, Tosoh), pre-equilibrated with buffer D (pH 8.2) and eluted with a linear NaCl gradient (0–2.0 M) at a flow rate of 0.3 ml min $^{-1}$. Fractions with high enzyme activity were combined and concentrated by ultrafiltration using a YM-50 membrane (Amicon). The concentrated fraction was subjected to gel filtration with a Sephacryl S-300 HR column (1.5 \times 45 cm; GE Healthcare) and pre-equilibrated with buffer D (pH 8.2) containing 0.2 M NaCl. The Pls purified to homogeneity was eluted at a constant flow rate of 0.15 ml min $^{-1}$. All purification steps were carried out at 4 °C.

Assay of A domain amino acid selectivity. The activation of proteinogenic amino acids and L-lysine analogs by the A domain of the Pls was investigated using standard ATP- 32 PP $_i$ exchange assays followed by liquid-scintillation counting⁷.

AMP-forming activity. The productions of AMP in reaction mixtures with L-lysine and its analogs were analyzed by HPLC (ref. 7). The relative activities were determined from the areas of the AMP peaks.

Structural analysis of ϵ -PL synthesized by Pls. A reaction mixture (120 μ l) consisting of 100 mM TAPS-NaOH buffer (pH 8.5), 2 mM L-lysine, 5 mM MgCl $_2$, 5 mM ATP, 1 mM DTT, 20% glycerol (v/v), 0.2% (w/v) NP-40 and 40 μ g ml $^{-1}$ Pls was incubated at 25 °C for 4 h. The reaction was quenched by heating at 100 °C for 5 min, then the mixture was centrifuged and the supernatant was concentrated to dryness *in vacuo*. When L-lysine methyl and ethyl esters were used as substrates, the reactions were quenched by addition of chloroform to avoid the ester hydrolysis of polymer products. To remove the glycerol, methanol-chloroform (1:1) was added, and a pellet containing the enzymatically synthesized ϵ -PL was obtained by centrifugation. This was dissolved in a small amount of 0.1 N HCl and analyzed by HPLC/ESI-MS (Esquire 4000, Bruker) using a C30 reversed-phase column (Develosil RPAQU-EOUS-AR-5; 150 \times 2.0 mm; Nomura). The ϵ -PL polymers were eluted with a linear gradient of acetonitrile (10–45% in 0.1% *n*-heptafluorobutyric acid (v/v)).

Chemical modification of ϵ -PL with DNP. This was performed as previously described¹⁵.

Determination of internal amino acid sequences of Pls. Tryptic digests of Pls were analyzed by HPLC/ESI-MS (Q-TOF2, Waters) using a standard protocol.

Cloning of the gene encoding Pls. Two sets of PCR primers were designed from the internal amino acid sequences pr-pp1F, pr-pp1R, pr-pp2F and pr-pp2R (**Supplementary Table 6** online). The PCR product obtained with one of the primer sets (pr-pp1F and pr-pp2R) was used as a probe to isolate the cosmid clone, pCOSpl, containing the *pls* gene from a genomic library of *S. albulus* NBRC14147 in the cosmid pOJ446 (ref. 16). The insert (~33 kilobase pairs) in pCOSpl was sequenced.

Inactivation of the *pls* gene. The open reading frame of the *pls* gene was amplified with the primers ϵ -PL-NRPS-F and ϵ -PL-NRPS-F (**Supplementary Table 6**) and inserted into the *Bam*HI and *Hind*III sites of pLAE003 (ref. 17). To inactivate *pls* in the resulting plasmid (pLAE $nrps$), a transposon carrying the *aac(3)IV* apramycin resistance gene¹⁸ was randomly inserted into pLAE $nrps$ via an *in vitro* transposon-insertion reaction with EZ-Tn5 transposase (Epicentre). Plasmid pLAE $nrps$ -apr, in which the transposon was inserted in *pls*, was subsequently introduced into the cryptic plasmid pNO33-curing *S. albulus* strain CR1 (ref. 9) by intergeneric conjugation with *E. coli* S17-1 (ref. 16). A *pls* gene disruptant (*S. albulus* CRM003) was isolated by a procedure described previously⁹.

Other methods. See **Supplementary Methods** online for remaining methods, including construction of the recombinant enzymes, assay for aminoacylation and performic acid oxidation¹⁹ of the radiolabeled Pls.

Accession codes. GenBank: The 33-kilobase-pair DNA fragment containing the *pls* gene from *S. albulus* NBRC14147 has been deposited under accession number AB385841.

Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website.

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AUTHOR CONTRIBUTIONS

Y.H., K.Y. and H.T. conceived and designed the experiments; Y.H., K.Y. and C.M. performed the experiments; and K.Y. and Y.H. wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturechemicalbiology/>.

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