Biochemistry and Enzymology of Poly-Epsilon-L-Lysine Biosynthesis

Yoshimitsu Hamano

Contents

Abstract *Streptomyces albulus* NBRC14147 (previously named IFO14147) is known to produce the amino-acid homopolymer antibiotic, poly-ε-L-lysine (ε-poly-L-lysine, ε-PL), consisting of 25–35 L-lysine residues with a linkage between the α-carboxyl group and the ε-amino group. Because ε-PL exhibits antimicrobial activity against a wide spectrum of microorganisms, including Gram-positive and Gram-negative bacteria, as well as antiphage activity, and because it is both safe and biodegradable, ε-PL has been introduced as a food preservative in Japan, South Korea,

Y. Hamano
Department of Bioscience, Fukui Prefectural University, 4-1-1 Matsuoka-Kenjojima, Eiheiji-cho, Fukui 910-1195, Japan
e-mail: hamano@fpu.ac.jp

Y. Hamano (ed.), *Amino-Acid Homopolymers Occurring in Nature*, Microbiology Monographs 15, DOI 10.1007/978-3-642-12453-2_2,

the United States, and other countries. This chapter covers the current knowledge and most recent advances in regard to the genetic system for *S. albulus* NBRC14147 and ε-PL synthetase.

1 Introduction

Streptomyces strains are known for their ability to synthesize commercially useful secondary metabolites having a wide range of biological activities. *Streptomyces albulus* NBRC14147 (previously named IFO14147) is known to produce the amino-acid homopolymer antibiotic, ε-PL, consisting of 25–35 L-lysine residues with a linkage between the α-carboxyl group and the ε-amino group (Fig. 1) (Shima and Sakai 1977; Shima and Sakai 1981a; Shima and Sakai 1981b). Because ε-PL exhibits antimicrobial activity against a wide spectrum of microorganisms, including Gram-positive and Gram-negative bacteria (Shima et al. 1984), as well as antiphage activity (Shima et al. 1982), and because it is both safe and biodegradable, ε-PL has been introduced as a food preservative in Japan, South Korea, the United States, and other countries.

The biological activity of ε-PL is known to be dependent on its molecular size. Shima and coworkers investigated the relationship between the molecular size of ε-PL and its antimicrobial activity against *Escherichia coli* K-12 (Shima et al. 1984). ε-PL with more than nine L-lysine residues severely inhibited microbial growth; however, the L-lysine octamer demonstrated negligible antimicrobial activity. In contrast, chemically synthesized α-poly-L-lysine that contains 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,

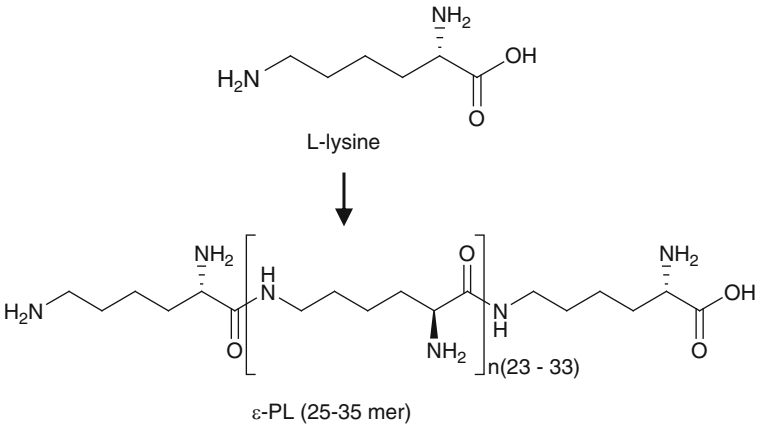

Fig. 1 Chemical structures of L-lysine and ε-PL (25-mer to 35-mer)

polymerization of L-lysine via an isopeptide bond is required to exert its biological activity, and the polymerization mechanisms involved in the chain-length diversity of ε-PL are of particular interest.

Investigation of an enzyme synthesizing ε-PL should facilitate biosynthetic engineering and help to create new classes of biopolymers. This review focuses on characterization of an ε-PL synthetase (Pls) and its biological machinery for ε-PL synthesis. In addition, an overview of effective genetic system for an ε-PL producer, *S. albulus* NBRC14147, which was used as a powerful tool for developing a deeper understanding of the Pls and for constructing an ε-PL overproducer, will be given.

2 Genetic System in an ε-PL Producer, *S. albulus* NBRC14147

Among the various gene transfer methods, polyethylene glycol (PEG)-mediated protoplast transformation is the standard for *Streptomyces* strains. In the initial PGE-mediated method transformation experiments on *S. albulus* NBRC14147, which were described for *Streptomyces lividans* (Kieser et al. 2000) and which used the typical cloning vectors having the pIJ101, pSG5, or pRES replicon (Kieser et al. 2000), no transformants were obtained. This unsuccessful outcome could be attributed to either or both of these reasons: (1) the vectors employed do not work in this strain; (2) the transformation methods are not suitable for direct application to the present strain of *S. albulus*. This section provides a brief overview of effective genetic system developed by Hamano et al.

2.1 Identification of the Cryptic-Plasmid pNO33 Replicon

A novel plasmid, pNO33, was detected in *S. albulus* NBRC14147 (Takagi et al. 2000). This large plasmid (37 kb) is a cryptic plasmid (Fig. 2), as none of its functions are yet known. To construct a cloning vector that can work in the *S. albulus* strain, the replicon (*Bcl*I–*Bam*HI 4.1 kb fragment, Fig. 2) of the cryptic plasmid pNO33 was used (Hamano et al. 2005). A circular plasmid carrying the *Bcl*I–*Bam*HI 4.1 kb fragment with the antibiotic (thiostrepton) resistant gene worked as a plasmid vector in *S. lividans* TK23, indicating that this plasmid (pBBH4) would also functionally operate as a replicon in *S. albulus*.

A database search with BLAST showed that the nucleotide sequence of this replicon had no similarity with those of the known replicons for cloning vectors, including pIJ702, which has the pIJ101 replicon and is frequently used in *Streptomyces* strains. Hamano et al. also reported that pBBH4 and pIJ702 are compatible for replication in the same cell of *S. lividans* (Hamano et al. 2005).

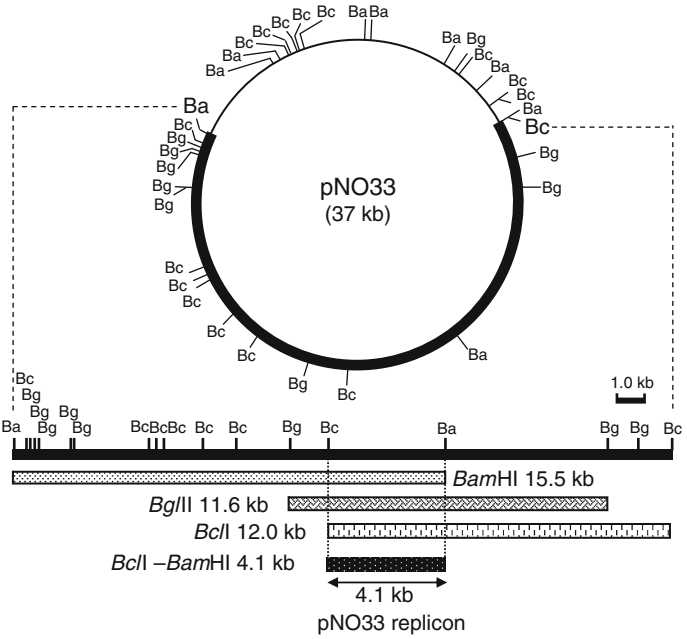

Fig. 2 Partial restriction map of pNO33 and schematic organization of the cloned fragment. *Bam*HI (15.5 kb), *Bgl*II (11.6 kb), and *Bcl*I (12 kb) fragments were cloned with a thiostrepton resistant gene (*Bcl*I 1.1 kb) in *S. lividans* TK23, demonstrating that these DNA fragments work as plasmid. Therefore, the pNO33 replicon is found to be located on the overlapping DNA fragment (*Bcl*I–*Bam*HI 4.1 kb). Abbreviations: Ba, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II

2.2 *Construction of the pNO33-Based Shuttle Vectors for* E. coli *and* Streptomyces *Strains*

Two cryptic-plasmid-based shuttle vectors, pLAE001 and pLAE003, were constructed for the PEG-mediated protoplast transformation and the intergeneric conjugation, respectively (Fig. 3) (Hamano et al. 2005). The plasmid pLAE001was constructed with pBBH4 and pNEB193 (commercially available plasmid vector for *E. coli*). The plasmid pLAE003 was constructed with pBBH4 and pK18mob.

In addition, a pNO33 curing strain of *S. albulus* NBRC14147, designated *S. albulus* CR1, was constructed and used as a host strain for the plasmids pLAE001 and pLAE003 (Hamano et al. 2005).

2.3 *PEG-Mediated Transformation of* S. albulus *CR1 Protoplast with pLAE001*

In PEG-mediated protoplast transformation, a transformation frequency of 10^2 transformants per 1 μg DNA was observed using pLAE001, which was prepared

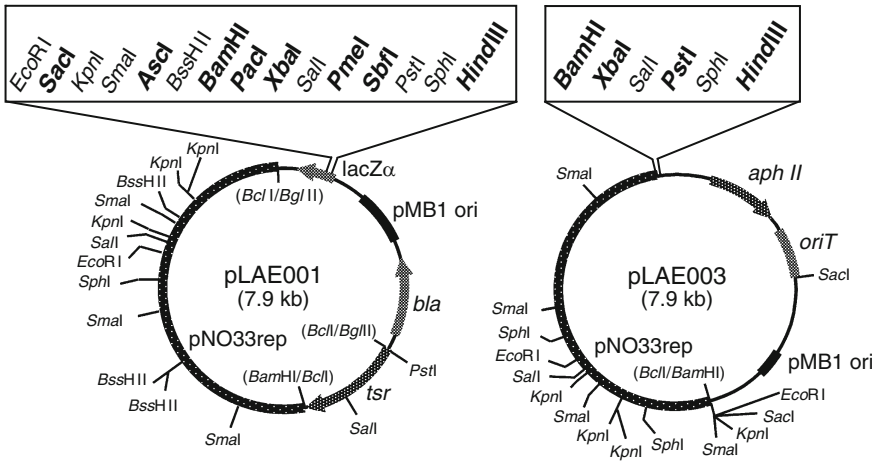

Fig. 3 pNO33-based shuttle vectors. pLAE001 and pLAE003 were used for the PEG-mediated protoplast transformation and intergeneric conjugation from *E. coli* to *S. albulus* CR1, respectively. Bold letters indicate cloning sites for restriction enzymes. Abbreviations: pNO33rep, pNO33 replicon (*Bcl*I–*Bam*HI 4.1 kb); *bla*, β-lactamase gene (Ampicillin resistance gene); *aph II*, aminoglycosides phosphotransferase II (neomycin/kanamycin resistance gene); *tsr*, thiostrepton resistance gene

from *S. lividans* TK23 (Hamano et al. 2005). When pLAE001 prepared from *S. albulus* CR1 was used for the transformation, the efficiency increased 100-fold, demonstrating that *S. albulus* CR1 shows a strong restriction barrier. In fact, the authors reported that pLAE001 prepared from a methylation-proficient *E. coli* strain, such as XL1-blue MRF′, cannot transform *S. albulus* CR1.

2.4 *Conjugal Transfer of the* oriT*-Vector, pLAE003, from* E. coli *to* S. albulus *CR1*

Recently, there has been considerable interest in the use of intergeneric conjugation as a means of gene transfer to bypass protoplast formation and regeneration (Mazodier et al. 1989; Matsushima et al. 1994; Flett et al. 1997; Voeykova et al. 1998; Paranthaman and Dharmalingam 2003; Choi et al. 2004; Stinchi et al. 2003). In *S. albulus* CR1, exconjugants were obtained employing a standard procedure, although the conjugation efficiency was very low (exconjugants per recipient spores: 9.0×10^{-8}). Nihira et al. have reported that the optimal concentration of $MgCl_2$ for conjugation differs among the various strains (Choi et al. 2004). In fact, in *S. albulus* CR1, the solid medium containing an optimal concentration of $MgCl_2$ (40 mM) provided the highest frequency (4.0×10^{-7}) (Hamano et al. 2005).

2.5 *Construction of a Genetically Engineered Strain of* S. albulus *CR1 for ε-PL Overproduction*

Shima et al. have reported that the L-lysine molecule is directly utilized in ε-PL biosynthesis (Shima et al. 1983). In most bacteria, L-lysine is biosynthesized by the amino-acid biosynthetic pathway from L-aspartic acid (aspartate pathway, Fig. 4). This pathway is also involved in the formation of other amino acids (i.e., L-methionine, L-isoleucine, and L-threonine). The first two enzymes in this pathway are aspartokinase (Ask) (EC.2.7.2.4), which catalyzes the phosphorylation of L-aspartic acid to produce L-4-phospho aspartic acid, and aspartate semialdehyde dehydrogenase (Asd) (EC.1.2.1.11), which reduces L-4-phospho aspartic acid into L-aspartate 4-semialdehyde. These two key enzymes are subject to complex regulation by the end-product amino acids. Because of the complexity of this pathway, different bacterial species have evolved diverse patterns of Ask regulation. For example, *E. coli* and *Bacillus subtilis* have three separate Ask isozymes, each controlled by one of the end products of the aspartate pathways, diaminopimelic acid, lysine, threonine, and methionine (Hitchcock et al. 1980; Theze et al. 1974; Zhang et al. 1990; Zhang and Paulus 1990). In contrast, only one Ask has been described in

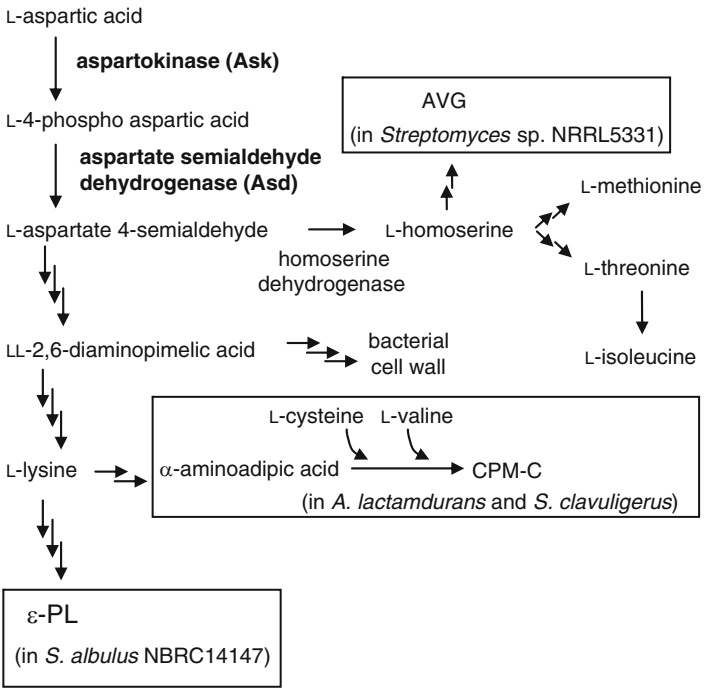

Fig. 4 The amino-acid biosynthetic pathway from L-aspartic acid (aspartate pathway). *AVG* aminoethoxyvinylglycine; *CPM-C* cephamycin C

antibiotic-producing actinomycetes. There are some reports of cloned *ask* genes from the rifamycin SV producer *Amycolatopsis mediterranei* (Zhang et al. 1999), from the cephamycin C (CPM-C, Fig. 4) producers *Amycolatopsis lactamdurans* (Hernando-Rico et al. 2001) and *Streptomyces clavuligerus* (Tunca et al. 2004), and from the aminoethoxyvinylglycine (AVG, Fig. 4) producer *Streptomyces* sp. NRRL5331 (Cuadrado et al. 2004). These studies have shown that regulatory mechanisms can differ even among the actinomycetes. Zhang et al. have reported inhibition of "*A. mediterranei*" Ask by L-lysine alone (Zhang et al. 2000), whereas Asks of *A. lactamdurans* and *S. clavuligerus* are feedback-regulated by the concerted action of L-lysine and L-threonine (Tunca et al. 2004; Hernando-Rico et al. 2001). Interestingly, these experimental observations indicate that the Asks of *A. lactamdurans* and *S. clavuligerus* (CPM-C producers) are slightly more resistant to concerted feedback-inhibition than Ask IIIs of *E. coli* (Ogawa-Miyata et al. 2001) and *B. subtilis* (Kobashi et al. 2001), although there is no discussion of this result in the respective reports. As such, the L-lysine productivity of these strains should be higher than those of *E. coli* and *B. subtilis*. This result could be due to the need for that, L-lysine, which is one of biosynthetic precursors of CPM-C, must be fully served to not only nascent protein biosynthesis but also the CPM-C biosynthesis (Fig. 4).

The ε-PL producer, *S. albulus* NBRC14147, can produce a large amount of ε-PL (usually 1–3 g/l). Therefore, Ask(s) of *S. albulus* was also expected to be potentially resistant to feedback-inhibition by L-lysine and/or L-threonine to provide sufficient amounts of L-lysine for ε-PL biosynthesis, similar to those of *A. lactamdurans* and *S. clavuligerus*. In fact, Hamano et al. demonstrated that the recombinant Ask of *S. albulus* NBRC14147 was found to be partially resistant to feedback-inhibition *in vitro* analysis (Hamano et al. 2007).

Hernando-Rico et al. have reported a construction of the feedback-resistant Ask of *A. lactamdurans* by substitutions of the two amino-acid residues, Ser^{301} and Gly^{345} (Hernando-Rico et al. 2001). However, a homologous expression of the mutated Ask has not been carried out due to the extreme difficulty in transforming *A. lactamdurans*. In *S. albulus* CR1, by random mutagenesis of the *ask* gene with error-prone PCR and the subsequent site-directed mutagenesis, Hamano et al. successfully constructed the mutated Ask, Ask (M68V), whose feedback-inhibition regulation was completely removed. Ask of *S. albulus* CR1 also has Ser^{301} and Gly^{345}, but no mutated Ask with substitutions of these two amino-acid residues were obtained. Rather, rAsk (M68V) was more appropriate for homologous expression in *S. albulus* CR1 to investigate whether L-lysine accumulation in the cell leads to ε-PL overproduction, as the calculated V_{max}^{Asp}/K_m^{Asp} values of rAsk (M68V) were ten-fold higher than that of the rAsk (wild type).

Using the genetic system for *S. albulus* CR1 described earlier, Hamano et al. constructed the genetically engineered strain of *S. albulus* CR1, which produced rAsk (M68V). Compared with the productivity in the use of Ask (wild), the homologous expression of Ask (M68V) predictably conferred a higher productivity of ε-PL (Fig. 5) (Hamano et al. 2007).

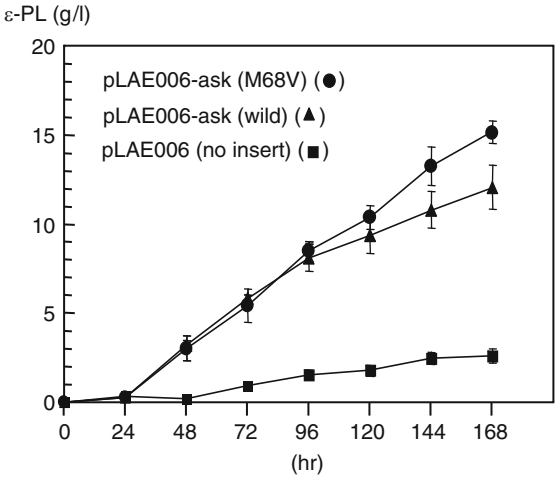

Fig. 5 The ε-PL productivities in the *S. albulus* CR1 strains expressing Ask (wild) or Ask (M68V). The *S. albulus* CR1 strains harboring pLAE006 (pLAE003 derivative harboring the *erm*E* promotor), pLAE006-ask (wild), and pLAE006-ask (M68V) were cultivated in ε-PL production medium containing 100 μg/ml neomycin by using a 5-l capacity bench scale jar-fermentor

3 ε-PL Synthetase

Two amino-acid "homopolymers" comprising a single type of amino acid are known in nature (Oppermann Sanio and Steinbuchel 2002): γ-poly-glutamic acid (γ-PGA) and ε-PL. The latter, which consists of 25–35 L-lysine residues with linkages between α-carboxyl groups and ε-amino groups (Fig. 1), exhibits antimicrobial activity against a spectrum of microorganisms including bacteria and fungi (Oppermann Sanio and Steinbuchel 2002; Shima et al. 1982; Shima et al. 1984). Due to its safety and biodegradability, it is used as a food preservative in several countries. The biological activity of ε-PL is known to be dependent on its molecular size. Shima and coworkers investigated the relationship between the molecular size of ε-PL and its antimicrobial activity against *E. coli* K-12 (Shima et al. 1984). ε-PL with more than nine L-lysine residues severely inhibited microbial growth; however, the L-lysine octamer demonstrated negligible antimicrobial activity. In contrast, chemically synthesized α-poly-L-lysine that contains 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 to exert its biological activity, and the polymerization mechanisms involved in the chain-length diversity of ε-PL are of particular interest.

Nonribosomal peptide synthetases (NRPSs) are multifunctional enzymes consisting of semiautonomous domains that synthesize numerous secondary metabolites (Walsh 2003; Schwarzer et al. 2003; Mootz et al. 2002; Marahiel et al. 1997). Using an assembly-line logic comprising multiple modules, they utilize 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 a 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 thiotemplate mechanism (Kawai et al. 2003; Saimura et al. 2008). However, as they 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. In addition, Hamano et al. recently identified and characterized ε-PL-degrading enzymes in an ε-PL producer, *S. albulus* NBRC14147 (Hamano et al. 2006). This raised the question of whether the degrading enzymes, rather than the biosynthetic machinery, might be responsible for generating products of diverse chain length. Actually, this section will reveal that the chain-length diversity of ε-PL is directly generated by the synthetase.

3.1 Purification of Pls from S. albulus *NBRC14147*

S. albulus NBRC14147 was grown to the late logarithmic phase, in which ε-PL production was observed. The mycelium 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, suggesting that Pls is an insoluble protein such as a membrane protein. Yamanaka et al. therefore solubilized this fraction with a nonionic detergent, Nonidet P-40 (NP-40) (Yamanaka et al. 2008). By successive purification steps including column chromatography, the solubilized Pls was finally purified 168-fold to apparent homogeneity. The relative molecular mass of the native enzyme as estimated by gel-filtration chromatography was 270 kDa. However, the molecular mass as estimated by denaturing SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was 130 kDa, suggesting that Pls is a homodimer.

3.2 Enzymatic Characterization of the Purified Pls

The purified enzyme was incubated with L-lysine and ATP. 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. 6). Incubation of Pls with L-lysine for different times did not change the relative amounts of each polymer produced during the polymerization reaction. ESI-tandem MS (ESI-MS/MS) analysis of the enzymatically synthesized polymer with 14 residues was identical to that of the reference standard polymer with 14 residues. Identical mass spectra to the reference standards were also observed for products of other chain lengths. Chemical modification of the synthesized polymers using 2,4-dinitrophenol (DNP) followed by TLC analysis against reference DNP-modified α- and ε-lysine polymers demonstrated that the α-amino groups of the L-lysine residues were labeled with DNP. This result shows that the polymer

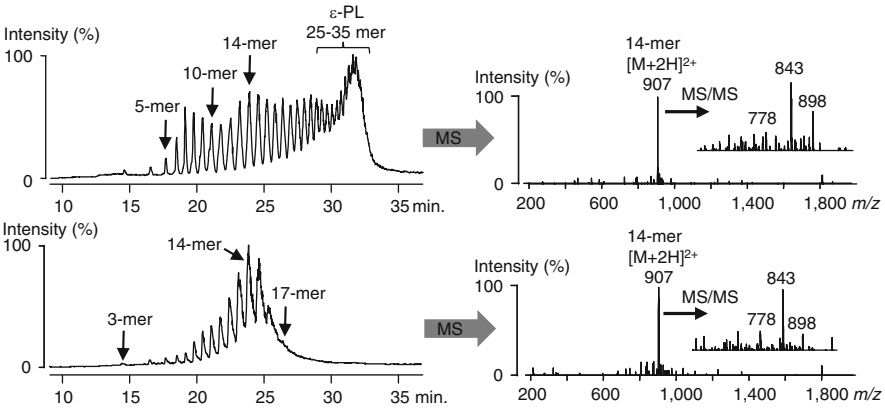

Fig. 6 Identification of the Pls reaction products. The reaction mixture (lower chromatogram and ESI mass spectrum) and a hydrolysate of ε-PL (upper chromatogram and ESI mass spectrum) were analyzed by HPLC/ESI-MS. The ε-PL hydrolysates (0.1 mg/ml) were prepared by hydrolysis with 1N HCl

consists of L-lysine residues with linkages between the α-carboxyl and ε-amino groups and confirms that the polydisperse polymer products are indeed ε-PL.

Yamanaka et al. investigated other enzymatic properties (Yamanaka et al. 2008). The enzyme required Mg^{2+}, ATP, 20–30% glycerol, 2 mM dithiothreitol, and 0.2–0.4% NP-40 for full activity. ATP was converted to AMP during the Pls reaction. No activity was detected with other nucleotides such as GTP, CTP, and TTP in the Pls polymerization reaction. Maximum activity occurs at an optimum pH of 8.5. The effect of temperature on the enzyme activity was investigated over the range of 10–45°C, with the maximum activity being observed at 25–30°C.

3.3 Cloning of the Gene Encoding Pls

To clone the gene encoding Pls, some of the amino-acid sequences of Pls were determined, and then PCR primers are designed. Using the PCR product as a probe, a 33-kbp DNA fragment containing the *pls* gene from *S. albulus* NBRC14147 was obtained. Interestingly, a BLAST database search showed that homologous genes are widely distributed among microorganisms. The *pls* gene encoded 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. The authors also constructed a knockout mutant of the putative *pls* gene using the genetic system described earlier in Sect. 3. The mutant produced no ε-PL, demonstrating that the cloned gene encodes Pls (Yamanaka et al. 2008).

With the protein sequence in hand, they 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) and subsequently loaded onto the 4′-phosphopantetheine (4′-PP) arm of the adjacent thiolation domain (T-domain) (Walsh 2003; Schwarzer et al. 2003; Mootz et al. 2002; Marahiel et al. 1997), 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. Their prior observation that AMP is released during the course of the reaction, along with the similarity of the ten residue sequence that confers substrate specificity in the putative Pls A-domain to that of the A-domain of the BacB (Challis et al. 2000) protein that adenylates L-lysine and the presence of Ser553 from the putative T-domain in proper alignment to be a 4′-PP-binding residue as compared with the T-domains of traditional NRPSs provides strong support for the classification of these regions of Pls as A- and T-domains. This distinguishes the mechanism of ε-PL biosynthesis from that of γ-PGA (Oppermann Sanio and Steinbuchel 2002), glutathione (Hibi et al. 2004), or cyanophycin (Oppermann Sanio and Steinbuchel 2002), which require phosphorylation of the carboxyl group.

Surprisingly, given the evidence for the presence of the A- 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. 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. Instead, a physicochemical analysis of the Pls amino-acid sequence with SOSUI (Hirokawa et al. 1998) suggested the existence of six transmembrane domains (TM-domains) surrounding three tandem soluble domains that display significant sequence similarity (with pairwise identities of 27, 22, and 23%) (Fig. 7). Alignment of the tandem domains with traditional C-domains demonstrated that the tandem domains did contain motifs showing similarity to His-motifs, or the HHxxxDG sequences found in all traditional C-domains, but the two histidine residues, known to be critical for catalysis, were not conserved. However, both the primary sequence and predicted three-dimensional structure of these domains showed similarity to acetyltransferases, which do show structural similarity to C-domains (Bergendahl et al. 2002; Keating et al. 2002). For this reason, Yamanaka et al. named the tandem sequences the C1-, C2-, and C3-domains, with the expectation that they would have a role in peptide bond formation (Yamanaka et al. 2008).

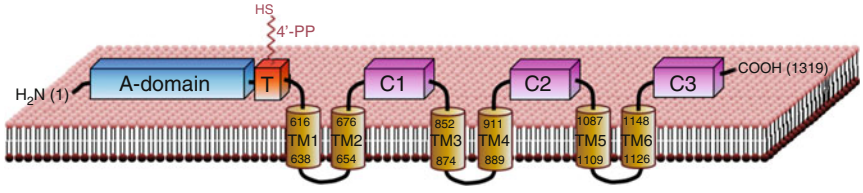

Fig. 7 Domain architecture of Pls. The A-, T-, six TM-domains, and three tandem domains (C1-, C2-, and C3-domain) are shown schematically. The numbers on Pls are the amino-acid residue numbers

3.4 Catalytic Mechanism of Pls

Yamanaka et al. explored the catalytic mechanism of the Pls using an ATP–PP_i exchange assay (Yamanaka et al. 2008). They observed Pls-mediated adenylation of L-lysine, but not any other proteinogenic amino acid (Fig. 8a). To investigate the function of the putative T-domain, they constructed a His-tagged recombinant Pls containing only the A- and T-domain (rPls-AT) using *S. lividans* TK23 as a heterologous host. Incubation of rPls-AT with L-[U-^{14}C]lysine and ATP as substrates resulted

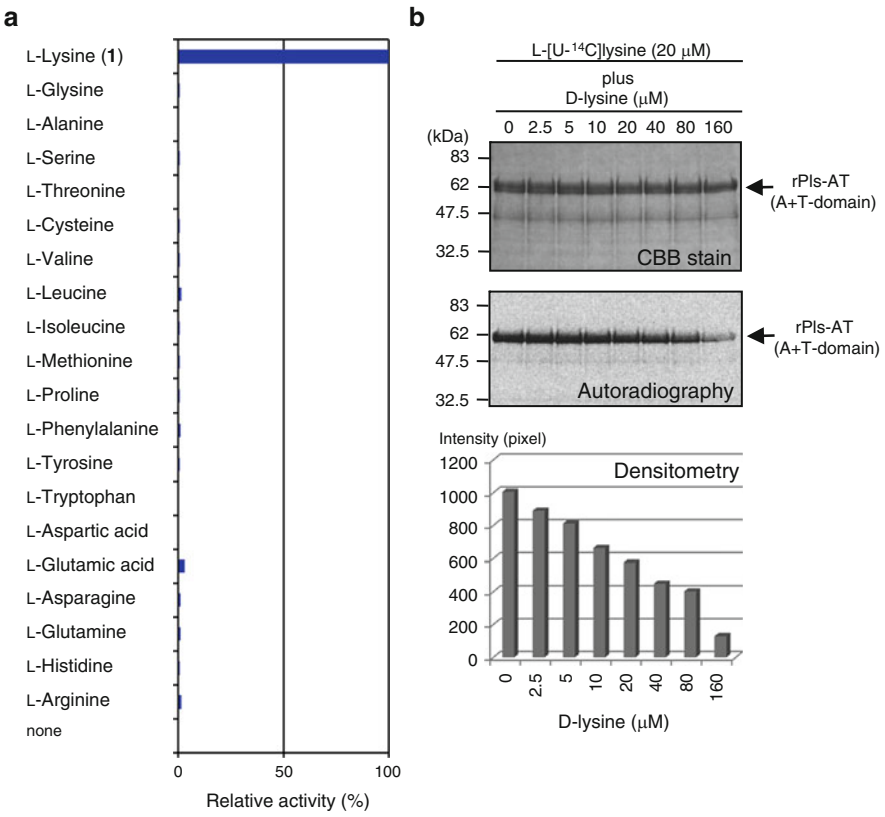

Fig. 8 Functional analysis of Pls. (**a**) The relative adenylation activities were determined on proteinogenic amino acids. Each value is represented as the mean of three experiments. (**b**) Aminoacylation of rPls-AT with L-[U-^{14}C]lysine was investigated. rPls-AT (Fig. 9) was incubated with L-[U-^{14}C]lysine plus D-lysine at 30°C for 12 h. The reaction mixtures were then subjected to SDS–PAGE (4 μg protein per lane). Proteins were stained with CBB R-250. The dried gel was exposed on an imaging plate and visualized by BAS-2500. The data from densitometry analysis of the autoradiography was shown. Data from this experiment is consistent with the fact that rPls-AT was modified with the 4′-PP cofactor in *S. lividans* TK23; the 4′-PP was posttranslationally transferred from Coenzyme A to the conserved serine residue (Ser553) of the T-domain by endogenous 4′-phosphopantetheinyl transferase(s), thus converting the inactive apo-form to its active holo-form

in loading of the labeled lysine onto the enzyme (Fig. 8b). However, ε-PL production was not detected, suggesting that the three tandem domains (C1-, C2-, and C3-domain) of Pls are essential for catalyzing the L-lysine polymerization reaction. To gain a better understanding of the function of the tandem domains, two additional recombinant Pls enzymes, rPls-ATC1C2 (lacking the C3-domain) and rPls-ATC1 (lacking both the C2- and C3-domain), were constructed. As observed for rPls-AT, which lacks all three C domains, no polymer products were detected for rPls-ATC1C2 and rPls-ATC1, suggesting 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-^{14}C] lysine and ATP. Analysis of the reaction mixture confirmed that Pls was radiolabeled during the reaction (Fig. 9a); however, performic acid treatment of the labeled Pls, which will release any small molecules attached via thioester bonds, only returned L-[U-^{14}C]lysine monomers instead of any polylysine chains (Fig. 9b). These results strongly suggested that the growing polymer products are not covalently attached to Pls during the polymerization reaction. Yamanaka et al. similarly observed that short-chain ε-PL oligomers (3-mer to 9-mer) are neither adenylated nor incorporated into polymers primed with free, deuterated L-lysine (L-Lys-d8); instead, Pls produced L-Lys-d8 homopolymers. To further test this mechanism, they employed L-lysine ethyl and methyl esters as substrates. HPLC/ESI-MS analysis of the reaction revealed that Pls produced the corresponding ethyl (Fig. 10) and methyl ester forms of ε-PL. While 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. 11), then the resulting L-lysine can be adenylated and loaded to the T-domain.

3.5 Substrate Specificity of Pls

Given that Pls can accept lysine esters as substrates, the substrate specificity of Pls was investigated further with seven L-lysine analogs (Fig. 12). L-Ornithine (L-Orn), L-kynurenine (L-KNR), and 3-amino-L-tyrosine (L-ATY) were not accepted as substrates. Pls was able to adenylate D-lysine (relative activity = 19%), but AMP-forming activity was not detected, suggesting 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 (Fig. 8b), in agreement with a model where D-lysine is stalled on the A-domain. Pls did successfully adenylate and pantetheinylate (as monitored by AMP formation) the rest of the analogs, including (5R)-5-hydroxy-L-lysine

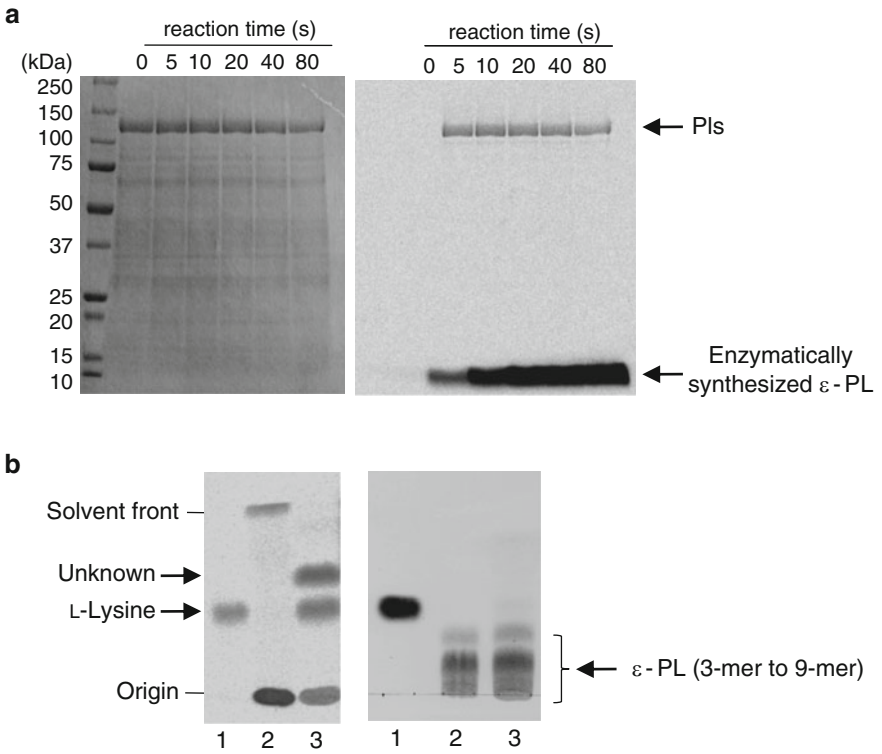

Fig. 9 Performic-acid oxidation of the radiolabeled Pls formed in the Pls reaction with L-[U-^{14}C]lysine. (**a**) After incubation of Pls with L-[U-^{14}C]lysine and ATP at 5°C for 0–80 s, the reaction mixtures were subjected to SDS–PAGE (5–20% gradient gel, 16 μg protein per lane). Proteins were stained 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 (lane 3) and formic acid (lane 2) were analyzed by silica gel thin-layer chromatography (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 (lane 2) and performic acids (lane3) and analyzed by silica gel TLC (image on the *right*). L-lysine (lane 1) was also used as the reference standards to confirm the mobility of the short-chain ε-PL oligomers. The samples were detected with ninhydrin reagent. In this oxidation experiments, an amino acid bound to enzyme as thioester is released from the enzyme not by formic acid but by performic acid. In the *left* image of panel (**b**), we detected the extra band (lane 3), which is known as the commonly observed unknown spots in performic oxidation experiments

(L-HLY; relative activity for adenylation, 43%; relative activity for AMP-formation, 6%), *O*-(2-aminoethyl)-L-serine (L-AES; corresponding values, 13 and 12%), and *S*-(2-aminoethyl)-L-cysteine (L-AEC; corresponding values, 5 and 6%). However, no homopolymers from these analogs were observed (see the lower chromatograms in Fig. 13b–d). In contrast, when a small amount of L-lysine was added to the reaction

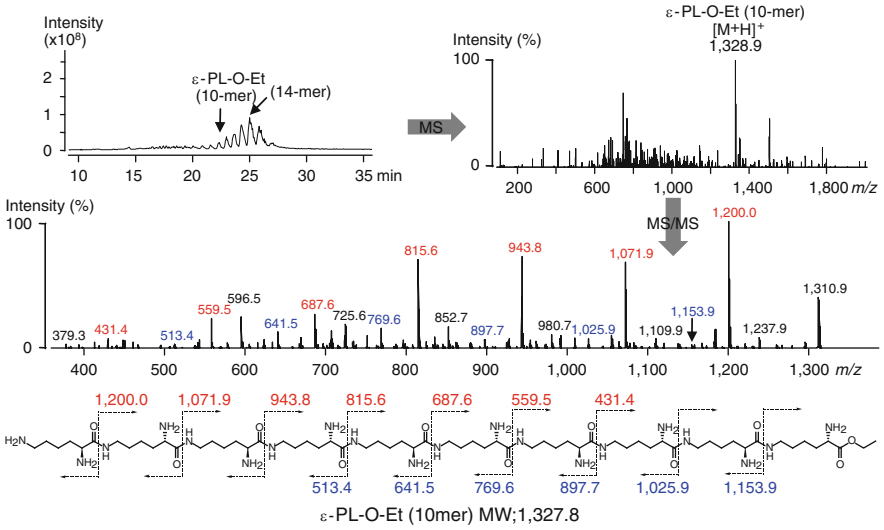

Fig. 10 Pls reaction with L-lysine ethyl ester. The Pls polymerization reaction with 2 mM L-lysine ethyl ester was performed and analyzed by HPLC/ESI-MS. The mass spectra and MS/MS spectra of the selected polymers (10-mer) in the reactions are shown

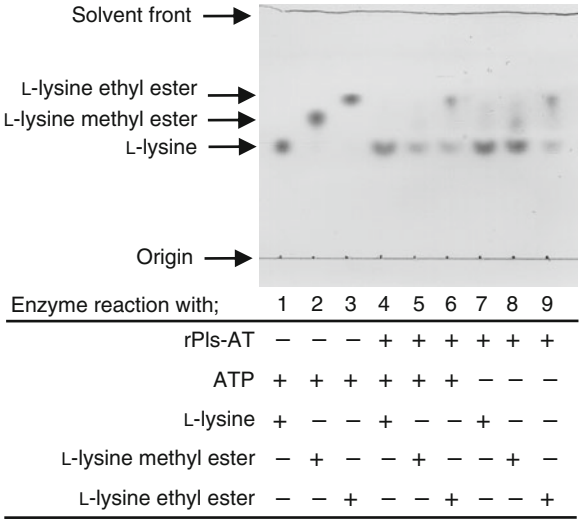

Fig. 11 Esterase activity in the A-domain. The rPls-AT reaction with 2 mM L-lysine, 2 mM L-lysine methyl ester, or 2 mM L-lysine ethyl ester was performed and analyzed by silica gel TLC. TLC was developed in 1-butanol/pyridine/acetic acid/H_2O, 2:1:1:2 (v/v). The products were detected with ninhydrin reagent. These results demonstrated that the esterase activities were not dependent on ATP. Addition, (+); No addition, (−)

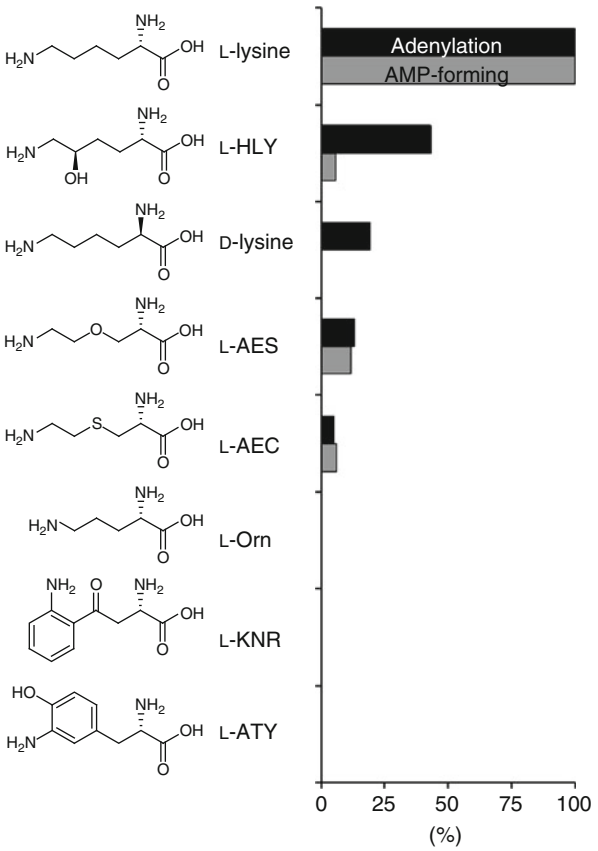

Fig. 12 Substrate specificities in the A- and T-domain of Pls. The relative activities in the adenylation and AMP-forming activities were determined on L-lysine and the L-lysine analogues. Each value is represented as the mean of three experiments. Error bars are not shown, because relative standard deviations of less than 5% were commonly calculated. L-HLY, (5R)-5-hydroxy-L-lysine; L-AES, *O*-(2-aminoethyl)-L-serine; L-AEC, *S*-(2-aminoethyl)-L-cysteine; L-Orn, L-ornithine; L-KNR, L-kynurenine; L-ATY, 3-amino-L-tyrosine

mixtures, heteropolymers consisting of L-lysine and the analogs were produced (see the upper chromatograms in Fig. 13b–d). These results indicate that the A- and T-domains are partially tolerant to substrate analogs, whereas the three tandem acetyltransferase domains show high specificity for L-lysine.

4 Concluding Remarks and Future Perspectives

In the ε-PL producing strain *S. albulus* NBRC14147, Hamano and coworkers successfully developed the genetic system, which was a powerful tool to construct a genetically engineered strain and to investigate the biosynthetic mechanism

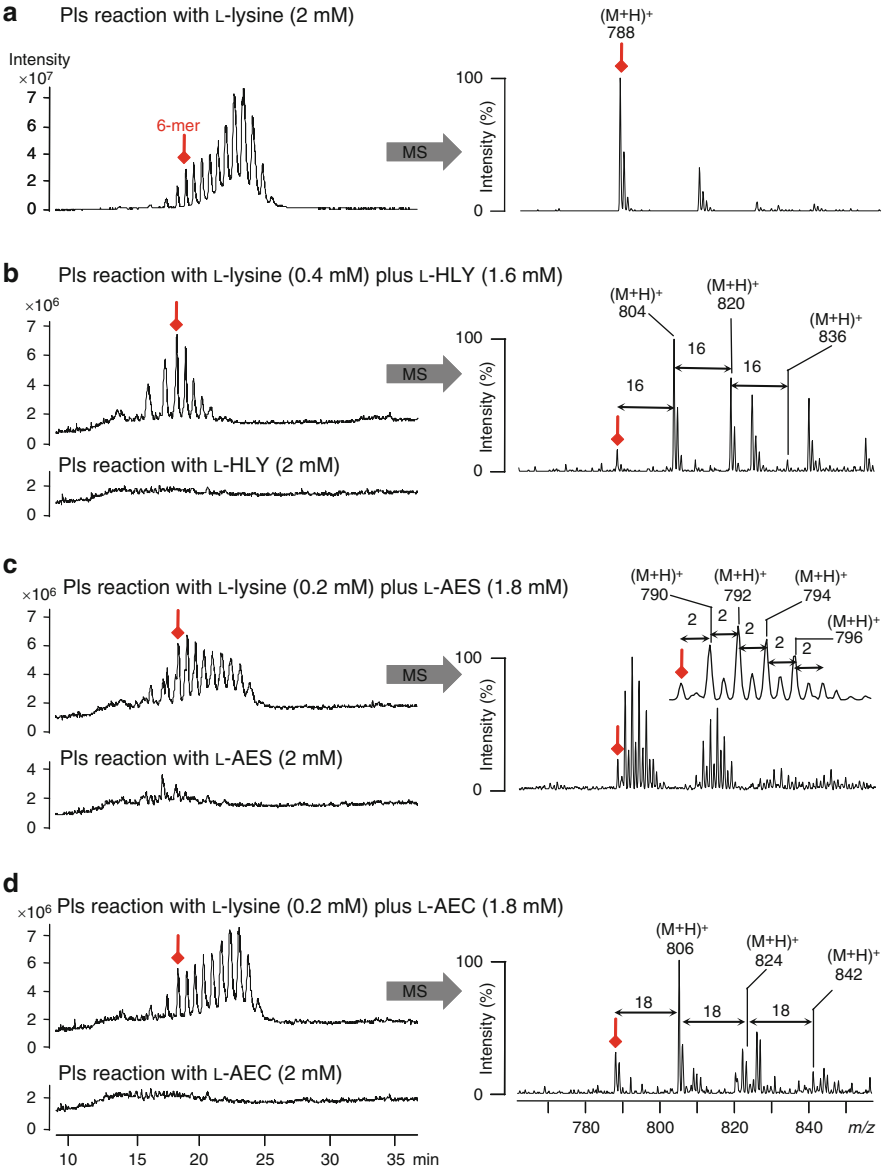

Fig. 13 *In vitro* production of heteropolymers consisting of L-lysine and the L-lysine analogues. The Pls reaction with 2 mM L-lysine (**a**), 1.6 mM L-HLY plus or minus 0.4 mM L-lysine (**b**), 1.8 mM L-AES plus or minus 0.2 mM L-lysine (**c**), or 1.8 mM L-AEC plus or minus 0.2 mM L-lysine (**d**) 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

of ε-PL. They also 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. Their explorations of Pls resulted in the development of a new model for the generation of chain-length diversity of ε-PL products, which integrates traditional NRPS logic, in the form of A- 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 N-terminus by the A- 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. 14). Since 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 their studies as ranging from 3 to 17 residues). This mechanism, in which a single polymer is created and then released (Fig. 14), is supported not only by the characterization of more basic Pls properties such as ATP turnover and ε-amine linkage formation but also by the observations that there was no difference in the relative amounts of each polymer during the polymerization reaction and that preexisting short chain polymers could not be incorporated into new chains (i.e., that 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. Yamanaka et al. 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.14).

In the analysis of the sequence of the three tandem domains, Yamanaka et al. identified a region reminiscent of the known His-motif from traditional C-domains, but lacking the histidine residues thought to be required for catalysis. It has been reported recently that the two histidine residues in the His-motif of VibH are also not critical for catalysis (Keating et al. 2002). As VibH, the functional C-domain of a vibriobactin NRPS, utilizes a similar mechanism to Pls – catalyzing peptide-bond formation between an NRPS-bound substrate, dihydroxybenzoate, and the freely diffusible substrate, norspermidin – these combined results may 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, the 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. With these findings in hand, it will be interesting to determine whether the activity of these degrading enzymes serves to create a shorter polymer with some defined function or simply regenerates the lysine

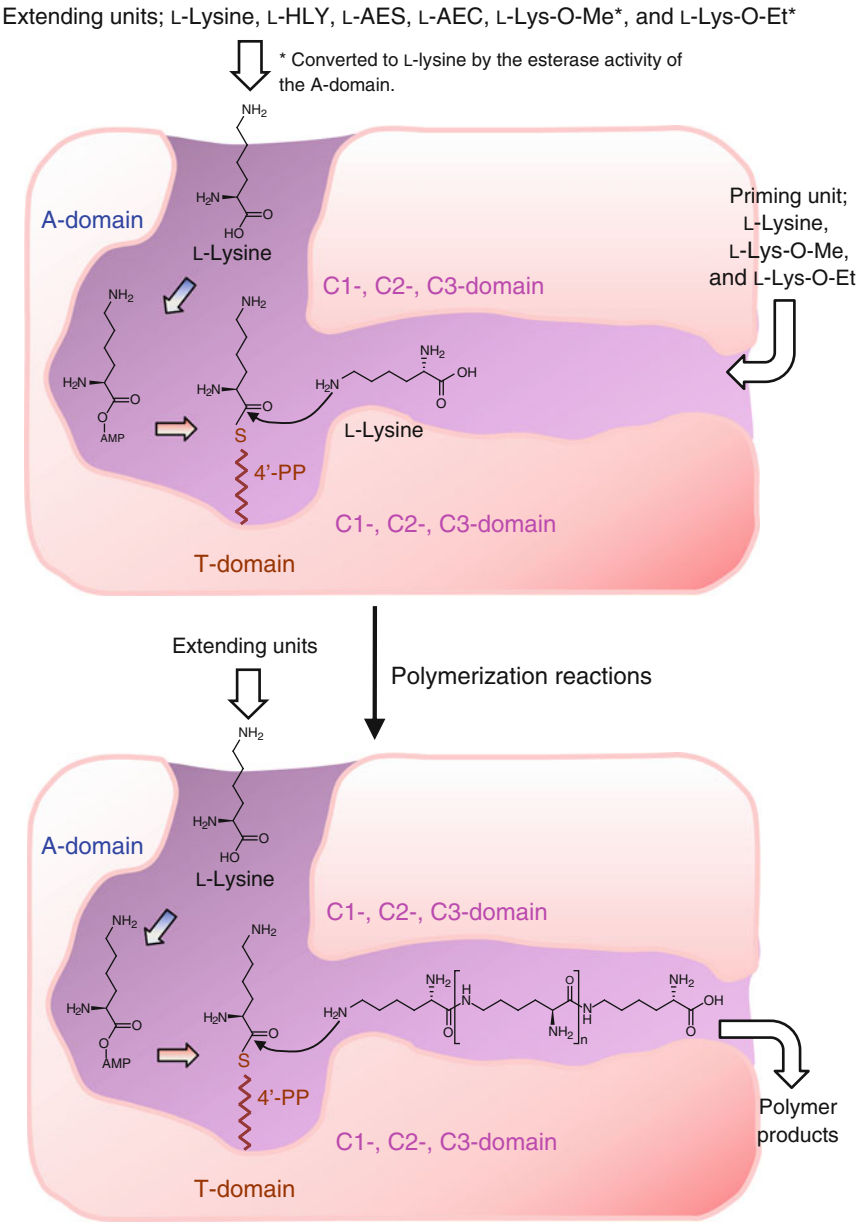

Fig. 14 Pls polymerization mechanism and the proposed model of the Pls catalytic cavity with substrate specificity. The clarified polymerization mechanism is shown schematically. L-Lys-O-Me, L-lysine methyl ester; L-Lys-O-Et, L-lysine ethyl ester

building blocks from the unusual ε-PL chain architecture. Similarly, further explorations of the homologous genes identified in our BLAST search should prove exciting, as their encoded proteins may synthesize amino-acid homopolymers other than ε-PL and γ-PGA. Investigations of these proteins as well as further exploration of Pls should facilitate biosynthetic engineering and help to create new classes of biopolymers.

References

Bergendahl V, Linne U, Marahiel MA (2002) Mutational analysis of the C-domain in nonribosomal peptide synthesis. Eur J Biochem 269:620–629

Challis GL, Ravel J, Townsend CA (2000) Predictive, structure-based model of amino acid recognition by nonribosomal peptide synthetase adenylation domains. Chem Biol 7:211–224

Choi SU, Lee CK, Hwang YI, Kinoshita H, Nihira T (2004) Intergeneric conjugal transfer of plasmid DNA from *Escherichia coli* to *Kitasatospora setae*, a bafilomycin B1 producer. Arch Microbiol 181:294–298

Cuadrado Y, Fernandez M, Recio E, Aparicio JF, Martin JF (2004) Characterization of the *ask-asd* operon in aminoethoxyvinylglycine-producing *Streptomyces* sp. NRRL 5331. Appl Microbiol Biotechnol 64:228–236

Flett F, Mersinias V, Smith CP (1997) High efficiency intergeneric conjugal transfer of plasmid DNA from *Escherichia coli* to methyl DNA-restricting streptomycetes. FEMS Microbiol Lett 155:223–229

Hamano Y, Nicchu I, Hoshino Y, Kawai T, Nakamori S, Takagi H (2005) Development of gene delivery systems for the ε-poly-L-lysine producer, *Streptomyces albulus*. J Biosci Bioeng 99:636–641

Hamano Y, Yoshida T, Kito M, Nakamori S, Nagasawa T, Takagi H (2006) Biological function of the *pld* gene product that degrades ε-poly-L-lysine in *Streptomyces albulus*. Appl Microbiol Biotechnol 72:173–181

Hamano Y, Nicchu I, Shimizu T, Onji Y, Hiraki J, Takagi H (2007) epsilon-Poly-L-lysine producer, *Streptomyces albulus*, has feedback-inhibition resistant aspartokinase. Appl Microbiol Biotechnol 76:873–82

Hernando-Rico V, Martin JF, Santamarta I, Liras P (2001) Structure of the *ask-asd* operon and formation of aspartokinase subunits in the cephamycin producer '*Amycolatopsis lactamdurans*'. Microbiology 147:1547–1555

Hibi T, Nii H, Nakatsu T, Kimura A, Kato H, Hiratake J, Oda J (2004) Crystal structure of gamma-glutamylcysteine synthetase: insights into the mechanism of catalysis by a key enzyme for glutathione homeostasis. Proc Natl Acad Sci USA 101:15052–15057

Hirokawa T, Boon Chieng S, Mitaku S (1998) SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics 14:378–379

Hitchcock MJ, Hodgson B, Linforth JL (1980) Regulation of lysine- and lysine-plus-threonine-inhibitable aspartokinases in *Bacillus brevis*. J Bacteriol 142:424–432

Kawai T, Kubota T, Hiraki J, Izumi Y (2003) Biosynthesis of ε-poly-L-lysine in a cell-free system of *Streptomyces albulus*. Biochem Biophys Res Commun 311:635–640

Keating TA, Marshall CG, Walsh CT, Keating AE (2002) The structure of VibH represents nonribosomal peptide synthetase condensation, cyclization and epimerization domains. Nat Struct Biol 9:522–526

Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) Practical Streptomyces Genetics. The John Innes Foundation, Norwich, UK

Kobashi N, Nishiyama M, Yamane H (2001) Characterization of aspartate kinase III of *Bacillus subtilis*. Biosci Biotechnol Biochem 65:1391–1394

Marahiel MA, Stachelhaus T, Mootz HD (1997) Modular peptide synthases involved in nonribosomal peptide synthesis. Chem Rev 97:2651–2673

Matsushima P, Broughton MC, Turner JR, Baltz RH (1994) Conjugal transfer of cosmid DNA from *Escherichia coli* to *Saccharopolyspora spinosa*: effects of chromosomal insertions on macrolide A83543 production. Gene 146:39–45

Mazodier P, Petter R, Thompson C (1989) Intergeneric conjugation between *Escherichia coli* and *Streptomyces species*. J Bacteriol 171:3583–3585

Mootz HD, Schwarzer D, Marahiel MA (2002) Ways of assembling complex natural products on modular nonribosomal peptide synthetases. Chembiochem 3:490–504

Ogawa-Miyata Y, Kojima H, Sano K (2001) Mutation analysis of the feedback inhibition site of aspartokinase III of *Escherichia coli* K-12 and its use in L-threonine production. Biosci Biotechnol Biochem 65:1149–1154

Oppermann Sanio FB, Steinbuchel A (2002) Occurrence, functions and biosynthesis of polyamides in microorganisms and biotechnological production. Naturwissenschaften 89:11–22

Paranthaman S, Dharmalingam K (2003) Intergeneric conjugation in *Streptomyces peucetius* and *Streptomyces* sp. strain C5: chromosomal integration and expression of recombinant plasmids carrying the *chiC* gene. Appl Environ Microbiol 69:84–91

Saimura M, Takehara M, Mizukami S, Kataoka K, Hirohara H (2008) Biosynthesis of nearly monodispersed poly(epsilon-L-lysine) in *Streptomyces* species. Biotechnol Lett 30:377–385

Schwarzer D, Finking R, Marahiel MA (2003) Nonribosomal peptides: from genes to products. Nat Prod Rep 20:275–287

Shima S, Sakai H (1977) Polylysine produced by *Streptomyces*. Agric Biol Chem 41:1807–1809

Shima S, Sakai H (1981a) Poly-L-lysine produced by *Streptomyces*. II. Taxonomy and fermentation studies. Agric Biol Chem 45:2497–2502

Shima S, Sakai H (1981b) Poly-L-lysine produced by *Streptomyces*. III. Chemical studies. Agric Biol Chem 45:2503–2508

Shima S, Matsuoka H, Sakai H (1982) Inactivation of bacteriophages by ε-poly-L-lysine produced by *Streptomyces*. Agric Biol Chem 46:1917–1919

Shima S, Oshima S, Sakai H (1983) Biosynthesis of ε-poly-L-lysine by washed mycelium of *Streptomyces albulus* No. 346. Nippon Nogeikagaku Kaishi 57:221–226

Shima S, Matsuoka H, Iwamoto T, Sakai H (1984) Antimicrobial action of ε-poly-L-lysine. J Antibiot (Tokyo) 37:1449–1455

Stinchi S, Azimonti S, Donadio S, Sosio M (2003) A gene transfer system for the glycopeptide producer *Nonomuraea* sp. ATCC39727. FEMS Microbiol Lett 225:53–57

Takagi H, Hoshino Y, Nakamori S, Inouye S (2000) Isolation and sequence analysis of plasmid pNO33 in the ε-poly-L-lysine-producing actinomycete *Streptomyces albulus* IFO14147. J Biosci Bioeng 89:94–96

Theze J, Margarita D, Cohen GN, Borne F, Patte JC (1974) Mapping of the structural genes of the three aspartokinases and of the two homoserine dehydrogenases of *Escherichia coli* K-12. J Bacteriol 117:133–143

Tunca S, Yilmaz EI, Piret J, Liras P, Ozcengiz G (2004) Cloning, characterization and heterologous expression of the aspartokinase and aspartate semialdehyde dehydrogenase genes of cephamycin C-producer *Streptomyces clavuligerus*. Res Microbiol 155:525–534

Voeykova T, Emelyanova L, Tabakov V, Mkrtumyan N (1998) Transfer of plasmid pTO1 from *Escherichia coli* to various representatives of the order Actinomycetales by intergeneric conjugation. FEMS Microbiol Lett 162:47–52

Walsh C (2003) Antibiotics: Action, Origins, Resistance. ASM Press, Washington DC

Yamanaka K, Maruyama C, Takagi H, Hamano Y (2008) Epsilon-poly-L-lysine dispersity is controlled by a highly unusual nonribosomal peptide synthetase. Nat Chem Biol 4:766–772

Zhang JJ, Paulus H (1990) Desensitization of *Bacillus subtilis* aspartokinase I to allosteric inhibition by meso-diaminopimelate allows aspartokinase I to function in amino acid biosynthesis during exponential growth. J Bacteriol 172:4690–4693

Zhang JJ, Hu FM, Chen NY, Paulus H (1990) Comparison of the three aspartokinase isozymes in *Bacillus subtilis* Marburg and 168. J Bacteriol 172:701–708

Zhang W, Jiang W, Zhao G, Yang Y, Chiao J (1999) Sequence analysis and expression of the aspartokinase and aspartate semialdehyde dehydrogenase operon from rifamycin SV-producing *Amycolatopsis mediterranei*. Gene 237:413–419

Zhang WW, Jiang WH, Zhao GP, Yang YL, Chiao JS (2000) Expression in *Escherichia coli*, purification and kinetic analysis of the aspartokinase and aspartate semialdehyde dehydrogenase from the rifamycin SV-producing *Amycolatopsis mediterranei* U32. Appl Microbiol Biotechnol 54:52–58

http://www.springer.com/978-3-642-12452-5

Amino-Acid Homopolymers Occurring in Nature
Hamano, Y. (Ed.)
2010, VII, 250 p. 62 illus., Hardcover
ISBN: 978-3-642-12452-5