AEM
Journals.ASM.org

ε-Poly-L-Lysine Peptide Chain Length Regulated by the Linkers Connecting the Transmembrane Domains of ε-Poly-L-Lysine Synthetase

Yoshimitsu Hamano,[a] Naoko Kito,[a] Akihiro Kita,[a] Yuuki Imokawa,[a] Kazuya Yamanaka,[b] Chitose Maruyama,[a] Hajime Katano[a]

Department of Bioscience, Fukui Prefectural University, Fukui, Japan[a]; Yokohama Research Center, JNC Corporation, Yokohama, Japan[b]

ε-Poly-L-lysine (ε-PL), consisting of 25 to 35 L-lysine residues with linkages between the α-carboxyl groups and ε-amino groups, is produced by *Streptomyces albulus* NBRC14147. ε-PL synthetase (Pls) is a membrane protein with six transmembrane domains (TM1 to TM6) as well as both an adenylation domain and a thiolation domain, characteristic of the nonribosomal peptide synthetases. Pls directly generates ε-PL chain length diversity (25- to 35-mer), but the processes that control the chain length of ε-PL during the polymerization reaction are still not fully understood. Here, we report on the identification of Pls amino acid residues involved in the regulation of the ε-PL chain length. From approximately 12,000 variants generated by random mutagenesis, we found 8 Pls variants that produced shorter chains of ε-PL. These variants have one or more mutations in two linker regions connecting the TM1 and TM2 domains and the TM3 and TM4 domains. In the Pls catalytic mechanism, the growing chain of ε-PL is not tethered to the enzyme, implying that the enzyme must hold the growing chain until the polymerization reaction is complete. Our findings reveal that the linker regions are important contributors to grasp the growing chain of ε-PL.

The homopoly(amino acid) ε-poly-L-lysine (ε-PL) is characterized by linkages between α-carboxyl groups and ε-amino groups of the L-lysine residues (Fig. 1). ε-PL was found in the culture filtrate of *Streptomyces albulus* no. 346 (currently designated *S. albulus* NBRC14147) (1–5). This strain extracellularly secretes ε-PL consisting of 25 to 35 L-lysine residues, and some different *Streptomyces* strains have been found to produce ε-PL with shorter chains (6–8). However, no ε-PL consisting of more than 36 residues has been found in nature. ε-PL (25- to 35-mer) is now commercially produced by a mutant of the NBRC14147 strain and is used as a food preservative in several countries for its antimicrobial activity against a spectrum of microorganisms, including bacteria and fungi. The biological activity of ε-PL depends on its molecular size. Shima and coworkers investigated this relationship in *Escherichia coli* K-12 (9) and showed that ε-PL with nine or more L-lysine residues severely inhibited microbial growth, whereas the L-lysine octamer demonstrated negligible antimicrobial activity.

We previously identified an ε-PL synthetase (Pls; Fig. 1) and reported the cloning of its gene (*pls*; GenBank accession no. AB385841) from *S. albulus* NBRC14147 (10–12). Pls is a membrane protein with an adenylation (A) domain and a thiolation (T) domain characteristic of the nonribosomal peptide synthetases (NRPSs). NRPSs are multifunctional enzymes consisting of catalytic domains (13–15). The amino acid substrate is activated as an aminoacyl-*O*-AMP by an A domain and subsequently loaded onto the 4′-phosphopantetheine (4′-PP) arm of the adjacent T domain with AMP release, thereby resulting in the formation of an aminoacyl-*S*-enzyme. A condensation (C) domain catalyzes a peptide bond formation between two amino acid substrates activated as the aminoacyl-*S*-enzyme. Thus, the C domain is crucial to the NRPS mechanism underlying peptide bond formation. However, Pls had no domain with significant sequence similarity to the traditional C domains. In addition, we found no thioesterase (TE) domain, which catalyzes the release of a final peptide product from the NRPS enzyme by hydrolysis to the free acid or cyclization to an amide or ester. Instead, Pls was suggested to have six transmembrane (TM) domains (TM1 to TM6) surrounding three tandem soluble domains that display sequence similarities with pairwise identities (10). The predicted three-dimensional structures of these three tandem domains showed similarity to those of acetyltransferases, which do show structural similarity to C domains. For this reason, we named the three tandem domains C1, C2, and C3. In a recent mutational analysis of these three domains using a recombinant Pls expression system (16), we demonstrated that the interconnected action of all three tandem domains is essential for peptide bond formation (17).

In vitro, Pls purified from the NBRC14147 strain produces ε-PL with a shorter chain (3- to 17-mer) when incubated with L-lysine and ATP (10). The catalytic mechanism is initiated in the N terminus by the A and T domains with the adenylation and transfer, respectively, of an incoming L-lysine monomer as an extending unit (Fig. 1). The C-terminal tandem domains (C1 to C3) catalyze the peptide bond formation between the extending unit and a freely diffusible L-lysine molecule (priming unit), producing an L-lysine dimer. The dimer is then used as a freely diffusible substrate (acceptor substrate) for the next polymerization reaction. Unlike the NRPS thiotemplate assembly logic, the growing peptide is not tethered to the enzyme. Thus, this catalytic cycle has no predetermined endpoint, and therefore Pls acts iteratively during ε-PL chain growth to obtain the chain length diversity of ε-PL.

In light of the production of the polydisperse ε-PL (3- to 17-mer) by Pls *in vitro*, the chain length diversity of ε-PL is directly

Received 10 April 2014 Accepted 31 May 2014
Published ahead of print 6 June 2014
Editor: M. Kivisaar
Address correspondence to Yoshimitsu Hamano, hamano@fpu.ac.jp.
Copyright © 2014, American Society for Microbiology. All Rights Reserved.
doi:10.1128/AEM.01201-14

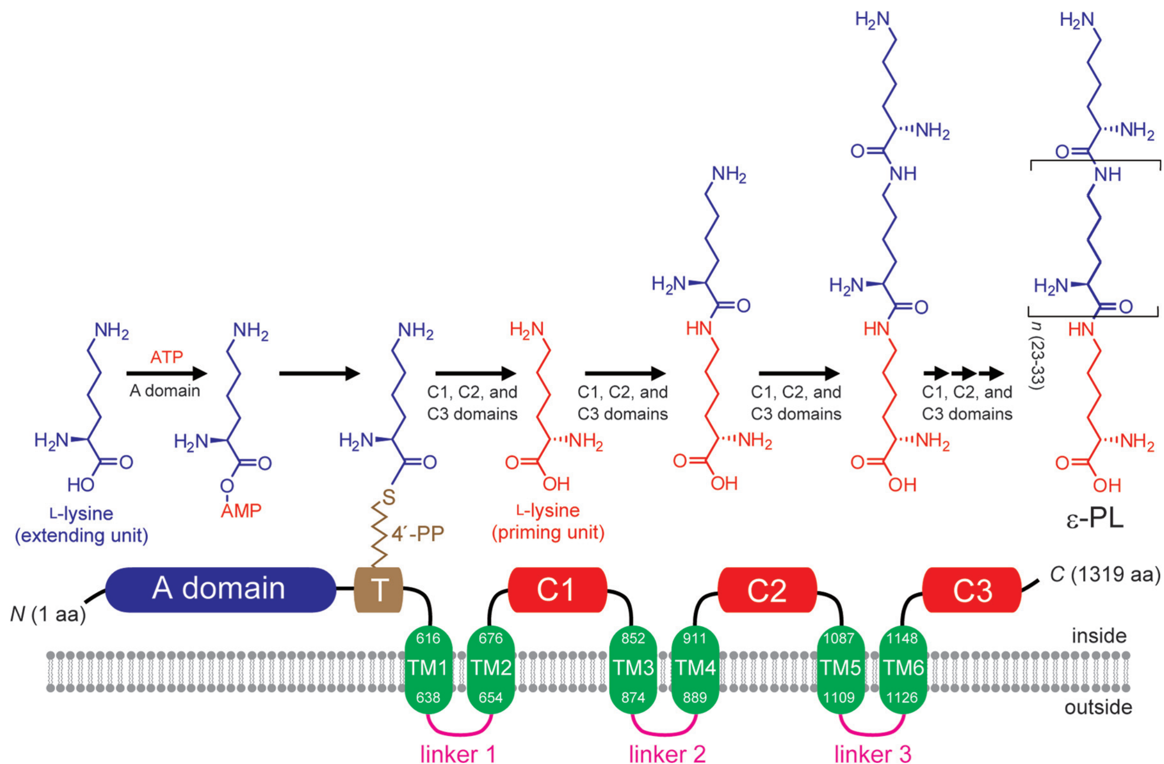

FIG 1 Domain architecture of Pls and schematics of catalytic mechanisms. The A domain, T domain, six transmembrane domains (TM1 to TM6), and three tandem domains (C1 to C3) are shown schematically. The numbers on Pls are the amino acid (aa) residue numbers. 4′-PP, 4′-phosphopantetheine.

generated by Pls rather than via the differential degradation of a uniform polymer by an ε-PL-degrading enzyme(s) that the ε-PL-producing strain produces. In fact, we recently reported that a mutant with a knockout of the *pld II* gene, which encodes an ε-PL-degrading enzyme that plays a central role in ε-PL degradation in *S. albulus* NBRC14147, was found to produce an ε-PL of unchanged polymer chain length (25- to 35-mer) (18). This finding revealed that *in vivo*, the length is determined not by ε-PL-degrading enzymes but rather by Pls itself and that the 25- to 35-mer is an original-chain-length polymer product that Pls synthesizes. However, the processes that control the chain length of ε-PL during the polymerization reaction are still not fully understood.

Here, we report on the identification of Pls amino acid residues involved in the regulation of ε-PL chain length. Our study, including a random mutation analysis of Pls, provides insight into the function of the linker regions connecting the TM domains in ε-PL chain length regulation.

MATERIALS AND METHODS

Chemicals. ε-PL was gifted from JNC Corp. (Tokyo, Japan). All other chemicals were of analytical grade.

Bacterial strains, plasmids, and media. *S. albulus* CRM003, in which the *pls* gene is inactivated (10), was used as a host strain for Pls expression experiments (16). The SLB medium and growth conditions used for *S. albulus* strains have already been described (19). S10.3 medium (20) was used for the cultivation of *Streptomyces lividans* TK23. *Escherichia coli* DH5α (TaKaRa, Otsu, Japan) was used for routine cloning, and *E. coli* S17-1 was used for intergeneric conjugation. The *E. coli*-*S. albulus* shuttle vector pLAE009 (Fig. 2) was used for the Pls expression experiments (16). *E. coli* strains harboring pLAE009 and pLAE009 derivatives were grown in

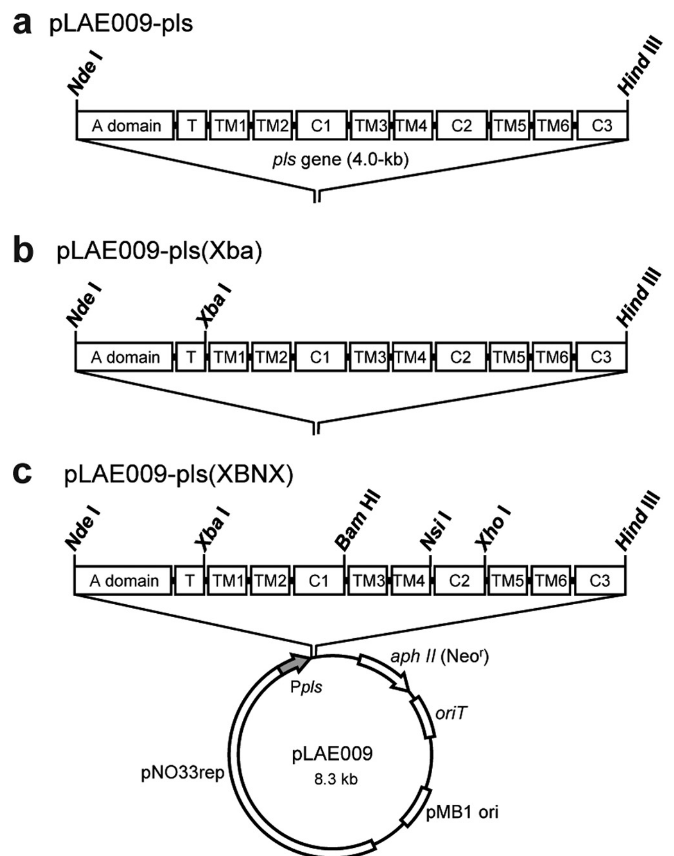

FIG 2 Plasmid maps of pLAE009-pls (a), pLAE009-pls(XbaI) (b), and pLAE009-pls(XBNX) (c).

TABLE 1 Oligonucleotides used in the site-directed mutagenesis experiments

Primer	Sequence
W646X-F	5′-CTCGGCCTGCTCGCCCCGCAGGTCNNSGCCCCGCACACCGCGTGGTGGCT-3′
W646X-R	5′-AGCCACCACGCGGTGTGCGGGGCSNNGACCTGCGGGGCGTGCAGGCCGAG-3′
L883X-F	5′-TTCCTGCTCCGCGACAGCGGCACCNNSGCCACAGCCGGGCTTCGCCTGCT-3′
L883X-R	5′-AGCAGGCGAAGCCCGGCTGTGGCSNNGGTGCCGCTGTCGCGGAGCAGGAA-3′

Luria-Bertani (LB) medium supplemented with neomycin (25 μg/ml). Recombinant DNA procedures for *E. coli* were performed using standard techniques as described by Sambrook and Russell (21). A Pls expression vector, pLAE009-pls, was used for the Pls expression experiments (Fig. 2) (16). In addition, pLAE009-pls(XbaI) and pLAE009-pls(XBNX) were used to randomize the *pls* gene (Fig. 2). The following microorganisms were used for MIC studies: *E. coli* W3110, *Bacillus subtilis* NBRC13169, *Staphylococcus aureus* FIR1169, *S. aureus* AB, and *Saccharomyces cerevisiae* S288C.

Randomization of the *pls* gene encoding the region from the TM1 domain to the C terminus by targeted PCR mutagenesis. To introduce mutations into the *pls* gene encoding the region from the TM1 domain to the C terminus, the XbaI-HindIII 2.2-kb fragment from pLAE009-pls(XbaI) (Fig. 2) was cloned into a vector, pBluescript SK(+). The resulting plasmid, pBluescript-pls(XbaI-HindIII), was subsequently used as a template for error-prone PCR (GeneMorph II random mutagenesis kit; Agilent Technologies, Santa Clara, CA). The amplified DNA fragment (*pls* gene$^{\text{TM1-Cterm}}$ library) with M13 forward and reverse primers was digested with XbaI and HindIII and was then ligated into pLAE009-pls(XbaI), which had been digested with the same restriction enzymes. The ligation mixture was used for the transformation of *E. coli* DH5α. Approximately 20,000 colonies of transformants were collected, and a *pls* gene$^{\text{TM1-Cterm}}$ plasmid library was prepared from the transformants.

S. lividans TK23, which is an ε-PL nonproducer, was employed as a screening host strain. The *pls* gene$^{\text{TM1-Cterm}}$ plasmid library was introduced into *S. lividans* TK23 using standard techniques as described by Kieser et al. (22). The resulting transformants (approximately 8,000) were individually inoculated into 300 μl of S10.3 medium supplemented with neomycin (50 μg/ml) and were cultivated until the stationary phase was reached (24 h at 30°C). Aliquots (7 μl) of the culture were transferred to 350 μl of M3G medium (ε-PL production medium) (18) supplemented with neomycin (50 μg/ml) and grown for 2 days at 30°C. For these cultivations, 96-deep-well plates were used and shaken at 2,000 rpm. After the culture broths were centrifuged, the production and molecular weight of ε-PL in the supernatants were analyzed qualitatively by tricine-SDS-PAGE to obtain transformants producing ε-PL with altered peptide chain length. ε-PL was stained with Coomassie brilliant blue (CBB) R-250 in tricine-SDS-PAGE analysis.

To confirm mutations in the screened *pls* genes, the plasmids were purified from the *S. lividans* TK23 transformants and sequenced. The plasmids were then introduced into a *pls* gene knockout strain, *S. albulus* CRM003, by intergeneric conjugation with *E. coli* S17-1 according to methods described previously (19). The resulting exconjugants were cultured in SLB medium (19) until the stationary phase was reached (24 h at 30°C). Aliquots (500 μl) of the culture were transferred to 50 ml of M3G medium supplemented with neomycin (50 μg/ml) for 4 days at 30°C. After the culture broths were centrifuged, ε-PL was purified from the supernatant according to the method described below. The peptide chain length of ε-PL was analyzed by high-performance liquid chromatography/electrospray ionization mass spectrometry (HPLC/ESI-MS) using a reversed-phase column (Sunniest RP-AQUA, 5 μm, 150 by 2.0 mm; ChromaNik Technologies, Osaka, Japan) at 30°C and a flow rate of 0.3 ml/min and with a gradient of acetonitrile in water containing 0.05% (vol/vol) *n*-heptafluorobutyric acid and 0.05% (vol/vol) formic acid run over 50 min (15 to 30% [vol/vol] acetonitrile for 10 min, 30 to 40% [vol/vol] acetonitrile for 30 min, and 90% [vol/vol] acetonitrile for 10 min).

Saturation mutagenesis of W646 and L883 in Pls. The codons encoding Pls amino acid residues W646 and L883 were replaced with the remaining 18 possible amino acids by a site-directed mutagenesis technique (QuikChange XL site-directed mutagenesis kit; Agilent Technologies) using the pBluescript-pls(XbaI-HindIII) plasmid as a template and two sets of degenerate primers: W646X-F and W646X-R for saturation mutagenesis of W646 and L883X-F and L883X-R for saturation mutagenesis of L883 (Table 1). The XbaI-HindIII 2.2-kb fragment carrying the mutations was ligated into pLAE009-pls(XbaI), which had been digested with the same restriction enzymes. The resulting plasmids were then introduced into *S. albulus* CRM003 and cultured in M3G medium for 4 days at 30°C by the same methods as described above. After the culture broths were centrifuged, the peptide chain length of ε-PL purified from the supernatants was analyzed by HPLC/ESI-MS under the same analytical conditions as described above. The ε-PL produced in the supernatant was quantified according to the method reported by Itzhaki (23).

Randomization of the *pls* gene encoding the linker region connecting the TM3 and TM4 domains by targeted PCR mutagenesis. To introduce mutations into the *pls* gene encoding the amino acid sequence region from the TM3 to the TM4 domain, the BamHI-NsiI 0.2-kb fragment from pLAE009-pls(XBNX) (Fig. 2) was cloned into a vector, pLITMUS28 (New England BioLabs, Ipswich, MA), and the resulting plasmid, pLITMUS28-pls(BamHI-NsiI), was used as a template for error-prone PCR. The amplified DNA fragment with M13 forward and reverse primers was digested with BamHI and NsiI and then ligated into pLAE009-pls(XBNX), which had been digested with the same restriction enzymes. The ligation mixture was used for the transformation of *E. coli* DH5α. Approximately 15,000 colonies of transformants were collected and used for the preparation of a *pls* gene$^{\text{TM3-TM4}}$ plasmid library. This library was introduced into *S. lividans* TK23, and the transformants (approximately 4,000) producing ε-PL with altered peptide chain lengths were screened by the same method as described above. The peptide chain length of ε-PL produced by *S. albulus* CRM003 strains expressing mutated Pls was investigated using the method described above.

Purification of ε-PL. The culture broth was centrifuged, and the supernatant was mixed with an equal volume of chloroform. After vigorous shaking, the aqueous layer was used for the further purification steps using tetraphenylborate anion (24).

Prediction of the TM domains in Pls homologues. The amino acid sequences of the TM domains were predicted by a physicochemical analysis using the SOSUI program (25).

RESULTS

Random mutagenesis of Pls and screening of variants producing ε-PL with altered peptide chain length. Our previous studies revealed that Pls directly generates the chain length diversity (25- to 35-mer) of ε-PL and that *in vivo*, the length is determined not by ε-PL-degrading enzymes but rather by Pls itself. However, little is known about the mechanism by which Pls controls its peptide chain length and diversity during the polymerization reaction. The N-terminal half (A and T domains) and the C-terminal half (TM1 domain to the C terminus) of Pls catalyze the activation and polymerization of the substrate L-lysine, respectively (Fig. 1). This also suggested that the peptide chain length of ε-PL is governed by one or more certain domains among the C-terminal half domains.

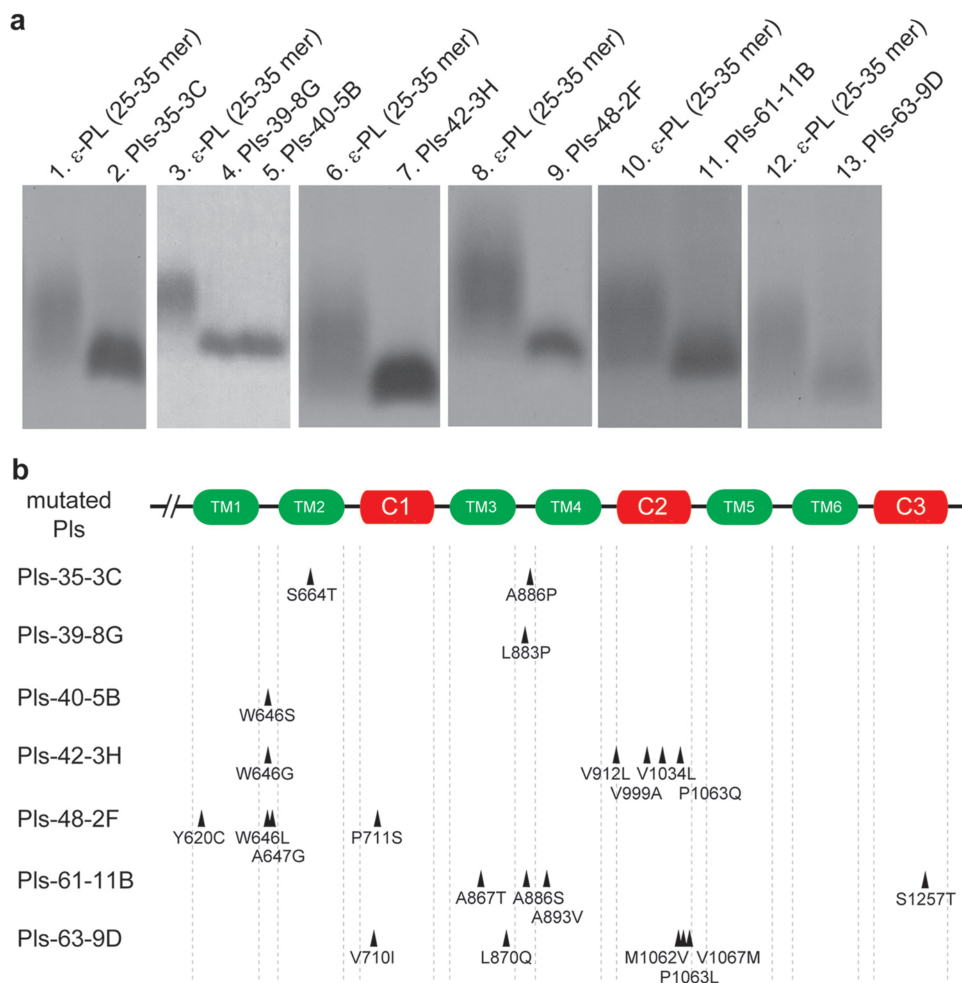

FIG 3 Pls variants producing short-chain ε-PL. (a) Tricine-SDS-PAGE analysis of ε-PL produced by seven Pls variants. The culture supernatants (10 μl) from the *S. lividans* TK23 transformants expressing the Pls variants Pls-35-3C (lane 2), Pls-39-8G (lane 4), Pls-40-5B (lane 5), Pls-42-3H (lane 7), Pls-48-2F (lane 9), Pls-61-11B (lane 11), and Pls-63-9D (lane 13) were subjected to tricine-SDS-PAGE. The ε-PL (25- to 35-mer, 30 μg/ml) solution (10 μl) was used as a control (lanes 1, 3, 6, 8, 10, and 12). ε-PL was stained with CBB R-250. (b) The mutation sites in the Pls variants are shown schematically.

In this study, therefore, we carried out a random mutagenesis analysis in the C-terminal half of Pls by targeted error-prone PCR (a *pls* gene$^{\text{TM1-Cterm}}$ library) to identify the domain(s) having such function.

For this experiment, we employed a heterologous host strain, *S. lividans* TK23, because the polyethylene glycol-assisted transformation of the CRM003 strain has a very low efficiency (19). Although the *S. lividans* TK23 transformant harboring pLAE009-pls or its derivatives (Fig. 2) produced ε-PL at low levels, tricine-SDS-PAGE analysis of the culture broth enabled us to detect such a small amount of the ε-PL produced in the supernatant of the culture broth and to analyze its molecular weight (data not shown). Using this method, we investigated the molecular weight of ε-PL produced by *S. lividans* TK23 transformants harboring the *pls* gene$^{\text{TM1-Cterm}}$ library. From approximately 8,000 transformants, we obtained 7 that produced ε-PL with shorter chains (Fig. 3a). However, no transformant producing a longer-chain ε-PL was obtained. To confirm mutations in the seven *pls* genes screened, the plasmids were purified from the transformants. The sequencing analysis revealed that the seven *pls* genes had one or more mutations in the region encoding the C-terminal half of Pls according to our experimental design. Interestingly, among the seven mutated enzymes, two variants, Pls-39-8G and Pls-40-5B, each had one amino acid substitution, L883P and W646S, respectively (Fig. 3b). These substitutions were found in two linker regions (linkers 1 and 2) connecting the TM1 and TM2 domains and the TM3 and TM4 domains (Fig. 1). Similarly, the other mutated enzymes had one or more mutations in these two linker regions, except for Pls-63-9D (Fig. 3b). These findings suggested that the linker regions connecting the TM domains in Pls are involved in the regulation of ε-PL chain length.

To investigate the precise chain lengths of ε-PL produced by Pls-39-8G (Pls-L883P) and Pls-40-5B (Pls-W646S), these two variants were expressed in the *S. albulus* CRM003 strain, and the peptide chain length of ε-PL produced was analyzed by HPLC/ESI-MS. Pls-L883P and Pls-W646S produced shorter chains of ε-PL, 10- to 23-mer and 9- to 23-mer, respectively, whereas the wild-type enzyme produced ε-PL consisting of 21 to 32 L-lysine residues in the present study (Fig. 4 and Table 2).

Saturation mutagenesis of W646 and L883 in Pls. Based on the alignment of Pls with its homologues (Fig. 5), W646 and L883 were found to be conserved. To gain a better understanding of

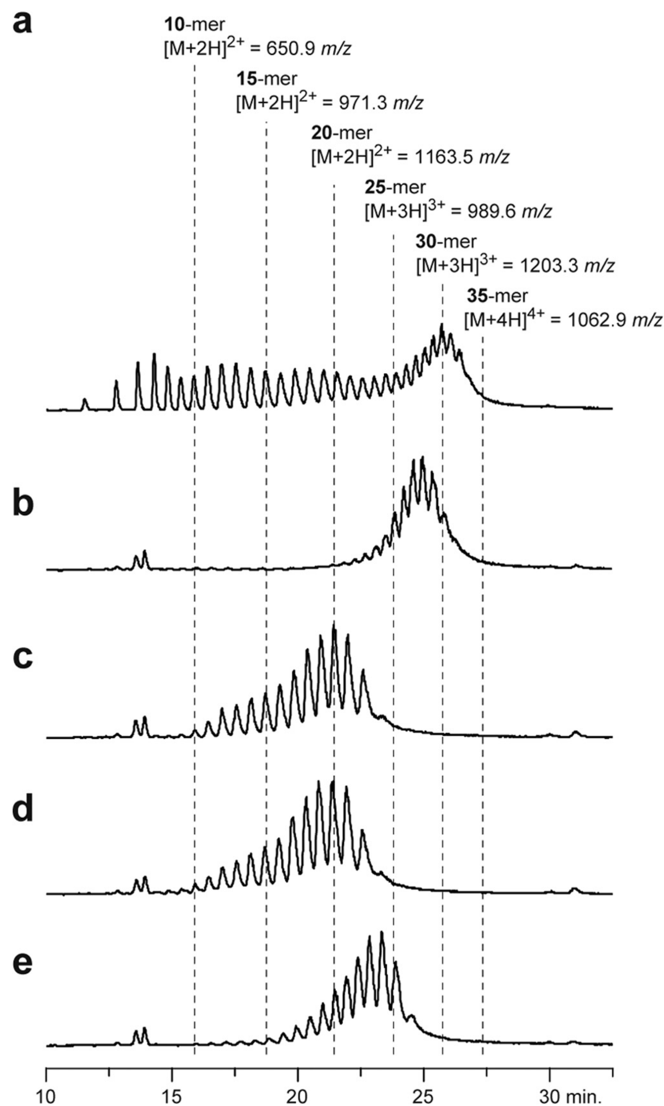

FIG 4 HPLC/ESI-MS analysis of ε-PL produced by the Pls variants. The ε-PL produced by the *S. albulus* CRM003 strains expressing the wild-type Pls (b) and Pls variants Pls-L883P (c), Pls-W646S (d), and Pls-S880I (e) were analyzed by HPLC/ESI-MS as described in Materials and Methods. To investigate the chain length of ε-PL produced by the variants, ε-PL hydrolysate (0.1 mg/ml) was prepared by hydrolysis with 1 N HCl and then analyzed by HPLC/ESI-MS (a).

these two amino acid residues, the codons encoding W646 and L883 were replaced with codons encoding the remaining 18 possible amino acids by site-directed mutagenesis. In the W646 residue, only substitutions for G, S, T, L, Y, D, and Q rendered the enzyme production of ε-PL (Table 2), and all of these mutated enzymes produced short-chain ε-PLs. In contrast, L883 residue was found to be more tolerant to substitutions (Table 2), and the chain lengths of all ε-PLs produced by the mutated enzymes were also shorter than that of ε-PL produced by the wild-type Pls. Thus, no mutated enzyme producing longer-chain ε-PL was found.

Mutation analysis of Pls by targeting the linker 2 connecting the TM3 and TM4 domains. The random mutation analysis of the C-terminal half of Pls suggested that linkers 1 and 2 were involved in the regulation of ε-PL chain length. However, considering the fact that W646 and L883 were conserved among the Pls homologues (Fig. 5a), the substitutions of these two amino acid residues could affect the structural stability of Pls, possibly producing ε-PL with an unnatural length. Linker 2, connecting the TM3 and TM4 domains, has fewer conserved amino acid residues (Fig. 5b). Therefore, in the present study, we further performed the mutation analysis of Pls by targeting linker 2 to explore the unconserved amino acid residue(s) that might be involved in the regulation of the ε-PL chain length. The *pls* gene$^{TM3\text{-}TM4}$ library was constructed and introduced into *S. lividans* TK23. Among approximately 4,000 transformants, 1 was found to produce a short-chain ε-PL according to tricine-SDS-PAGE analysis (data not shown). Sequencing analysis of the plasmid obtained from the transformant revealed one amino acid substitution, S880I, in Pls (Fig. 5b). To investigate the precise chain length of ε-PL produced by Pls-S880I, the mutated enzyme was expressed in the *S. albulus* CRM003 strain, and the peptide chain length of ε-PL produced was analyzed by HPLC/ESI-MS. Pls-S880I produced a shorter chain (15- to 27-mer) of ε-PL (Fig. 4e).

Antibiotic activities of short-chain ε-PLs. We investigated the antibiotic activities of ε-PL (9- to 23-mer) produced by Pls-W646S and of ε-PL (15- to 27-mer) produced by Pls-S880I against microorganisms (Table 3). Interestingly, ε-PL (15- to 27-mer) showed higher levels of activity than those of the naturally occurring ε-PL (25- to 35-mer) against *E. coli* W3110, *S. aureus* FIR1169, and *S. cerevisiae* S288C among the microorganisms tested. Interestingly, ε-PL (9- to 23-mer) showed the highest activity against *S. cerevisiae* S288C.

DISCUSSION

The C-terminal half (TM1 domain of the C terminus) of Pls catalyzes the polymerization of the substrate L-lysine (Fig. 1). We therefore speculated that the peptide chain length of ε-PL is governed by one or more certain domains among the TM domains and C1 to C3 domains. Contrary to our expectation, the present study demonstrated that the linker regions connecting the TM1 and TM2 domains and the TM3 and TM4 domains in Pls were involved in the regulation of the ε-PL chain length. For this study, we developed a two-step screening strategy to obtain mutated Pls producing ε-PL with altered peptide chain lengths. In the first step, we employed the heterologous host strain *S. lividans* TK23, which shows a high transformation efficiency, to introduce the *pls* gene mutation library, although the TK23 transformants did not produce ε-PL in amounts suitable for HPLC/ESI-MS analysis. To investigate the precise chain length of ε-PL by HPLC/ESI-MS as a second screening step, the mutated Pls found in the first screening step was expressed in the homologous host strain *S. albulus* CRM003, in which the *pls* gene is inactivated. We recently described the expression of the C-terminal His-tagged recombinant Pls (rPls) in the strain CRM003 (16). However, the purified rPls had been found to produce a shorter-chain ε-PL (3- to 17-mer) *in vitro*. We therefore used the ε-PL produced *in vivo* to investigate the chain length.

Although no Pls variant producing longer-chain ε-PL was obtained, we finally found eight Pls variants that produced shorter chains of ε-PL (Fig. 3 and 4). The findings for Pls-W646S, Pls-S880I, and Pls-L883P revealed the participation of two linker regions connecting the TM1 and TM2 domains and the TM3 and TM4 domains in the regulation of ε-PL chain length. However, it is unclear whether another linker (linker 3) connecting the TM5

TABLE 2 Saturation mutagenesis of W646 and L883 in Pls

Pls-W646X mutation[a]	ε-PL produced by Pls-W646X: Productivity (μg/ml)[b]	ε-PL produced by Pls-W646X: Chain length (-mer)[c]	Pls-L883X mutation[a]	ε-PL produced by Pls-L883X: Productivity (μg/ml)[b]	ε-PL produced by Pls-L883X: Chain length (-mer)[c]
W646G	166	9–23	L883G	161	10–21
W646A	0	NA	L883A	0	NA
W646S	163	9–23 (Fig. 4d)	L883S	162	10-21
W646T	166	9–23	L883T	165	10–22
W646C	0	NA	L883C	0	NA
W646V	0	NA	L883V	0	NA
W646L	166	9–23	None (wild type)	173	21–32 (Fig. 4b)
W646I	0	NA	L883I	167	19–28
W646 M	0	NA	L883 M	0	NA
W646P	0	NA	L883P	163	10–23 (Fig. 4c)
W646F	0	NA	L883F	169	18–28
W646Y	169	9–25	L883Y	167	10–25
None (wild type)	173	21–32 (Fig. 4b)	L883W	165	10–25
W646D	168	9–23	L883D	160	9–21
W646E	0	NA	L883E	170	11–25
W646N	0	NA	L883N	0	NA
W646Q	164	9–23	L883Q	167	10–23
W646H	0	NA	L883H	0	NA
W646K	0	NA	L883K	162	9–21
W646R	0	NA	L883R	165	9–22

[a] The mutated enzymes were expressed using the expression vector pLAE009 and the host strain *S. albulus* CRM003.
[b] Each value represents the mean of results of two experiments.
[c] The peptide chain lengths of the PL produced by the mutated enzymes were analyzed by HPLC/ESI-MS. NA, not applicable.

and TM6 domains was involved in this regulation. Pls-63-9D, which has no mutation in the linker regions, also produced the short-chain ε-PL (Fig. 3). Probably, the substitution of L870, which is conserved in Pls homologues (Fig. 5) and is close to linker 2, affected the conformation of linker 2, thereby producing the short chain. This also suggested that the mutations of W646 and L883, which are also conserved in Pls homologues (Fig. 5), affected the conformation of the linkers. Similarly, Pls-42-3H and Pls-48-2F (Fig. 3) have the mutation of the conserved W646 residue, and Pls-61-11B also has the mutation of the conserved A886 residue. Thus, these conserved amino acid residues seem not to interact directly with the growing chain of ε-PL by electrostatic interaction, hydrophobic interaction, or hydrogen bonding interaction. This hypothesis is supported by the fact that the saturation mutagenesis of W646 and L883 residues showed no obvious correlations between the amino acid properties (charge, polarity, aro-

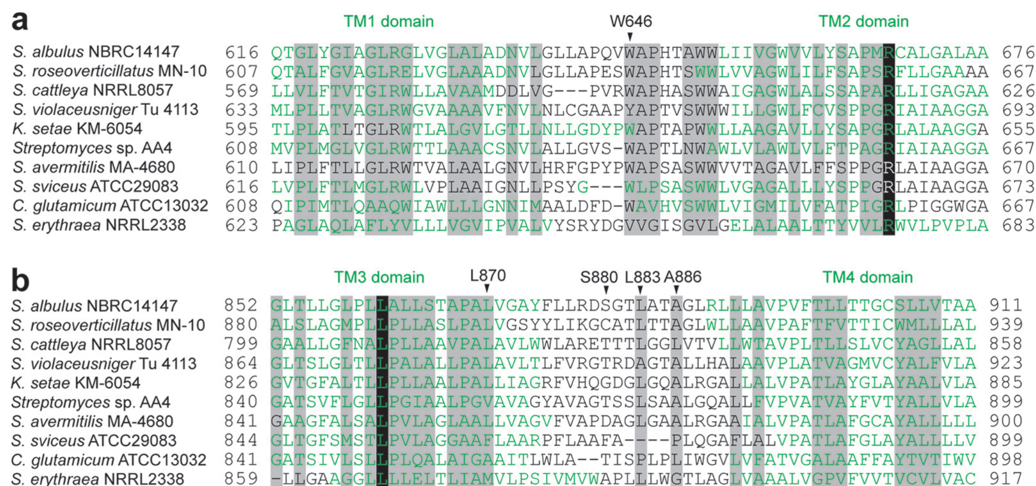

FIG 5 Alignment of the linker regions in Pls homologues. The amino acid sequences of the flanking regions of linker 1 (a) and linker 2 (b) were aligned with those of the corresponding linkers from Pls homologues. The amino acid sequences of the TM domains in Pls and its homologues were predicted by a physicochemical analysis using the SOSUI program and are shown in green. Areas shaded dark gray and light gray indicate identical and similar amino acid residues, respectively. The locations of the five important amino acid residues (W646, L870, S880, L883, and A886) of Pls are indicated. The following amino acid sequences were obtained from the NCBI database: those of the Pls homologues from *Streptomyces roseoverticillatus* MN-10 (GenBank accession number BAH85292), *Streptomyces cattleya* NRRL 8057 (NCBI reference number YP_004920722), *Streptomyces violaceusniger* Tu 4113 (YP_004816057), *Kitasatospora setae* KM-6054 (YP_004907834), *Streptomyces* sp. strain AA4 (WP_009079546), *Streptomyces avermitilis* MA-4680 (NP_827809), *Streptomyces sviceus* ATCC 29083 (EDY59523), *Corynebacterium glutamicum* ATCC 13032 (NP_601907), and *Saccharopolyspora erythraea* NRRL 2338 (YP_001105424).

TABLE 3 Antibiotic activities of the short-chain ε-PLs

Microorganism	MIC (μg/ml)[a]		
	ε-PL (25- to 35-mer)[d]	ε-PL (9- to 23-mer)[e]	ε-PL (15- to 27-mer)[e]
Escherichia coli W3110[b]	3.1	3.1	1.6
Bacillus subtilis NBRC13169[b]	0.8	0.8	0.8
Staphylococcus aureus FIR1169[b]	12.5	50.0	6.3
Staphylococcus aureus AB[b]	12.5	50.0	12.5
Saccharomyces cerevisiae S288C[c]	400.0	100.0	200.0

[a] MICs were determined by the broth dilution method. Two independent experiments yielded identical values.
[b] Cultivated in heart infusion broth (Difco) medium.
[c] Cultivated in yeast-peptone-dextrose.
[d] Gifted from JNC Corp. (Tokyo, Japan).
[e] Purified by the method using tetraphenylborate anion and used for MIC study.

maticity, size, and electronic properties) and the resulting ε-PL chain length (Table 2). On the other hand, the mutation of the unconserved S880 resulted in the alteration of the ε-PL chain length (Fig. 4e), suggesting that S880 might directly interact with the growing chain of ε-PL. In the Pls catalytic mechanism, the growing chain of ε-PL is not tethered to the enzyme. This implies that the enzyme must hold the growing chain until the polymerization reaction is complete. Although the underlying mechanism is still unclear, our findings reveal that the linker regions are important contributors to the retention of the growing chain of ε-PL.

Short-chain ε-PLs are known to show unique antibiotic activities (6), although a chain length with nine or more L-lysine residues is required to inhibit microbial growth (9). In fact, ε-PL (15- to 27-mer) obtained in the present study seemed to show a higher activity level than that of the naturally occurring ε-PL (25- to 35-mer) (Table 3). To date, some microorganisms producing short-chain ε-PLs have been identified, but their abilities to produce ε-PL were found to be low (6). In the present study, the mutated Pls enzymes produced short-chain ε-PLs at the same levels as the wild type (Table 2). Thus, mutating the linker regions connecting the TM domains should be a good strategy for effective production of short-chain ε-PL. In addition to this strategy, it is known that the use of ε-PL production media containing polyols such as glycerol, ethylene glycol, and trimethylene glycol resulted in the shortening of the ε-PL chain length (6, 26); the *S. albulus* NBRC14147 strain produced a short-chain ε-PL (13- to 28-mer) in the ε-PL production medium containing 2.5% pentamethylene glycol (1,5-pentanediol). However, the polymer was produced as an ester formed between the terminal carboxyl group of ε-PL and a hydroxyl group of pentamethylene glycol. The engineering of the Pls linker regions will be a powerful tool for further production of different short-chain ε-PLs without ester formation. This is the first example, to our knowledge, of the shortening of ε-PL chain length by Pls engineering.

ACKNOWLEDGMENTS

This work was supported, in part, by the Japan Society for the Promotion of Science (JSPS), KAKENHI grant numbers 20780057 (to Y.H.) and 25660064 (to Y.H.), and by the Industrial Technology Research Grant Program in 2008 of the New Energy and Industrial Technology Development Organization (NEDO) of Japan (grant to Y.H.).

REFERENCES

1. **Shima S, Sakai H.** 1981. Poly-L-lysine produced by Streptomyces. Part II. Taxonomy and fermentation studies. Agric. Biol. Chem. **45:**2497–2502.
2. **Oppermann-Sanio FB, Steinbüchel A.** 2002. Occurrence, functions and biosynthesis of polyamides in microorganisms and biotechnological production. Naturwissenschaften **89:**11–22. http://dx.doi.org/10.1007/s00114-001-0280-0.
3. **Shima S, Sakai H.** 1977. Polylysine produced by *Streptomyces*. Agric. Biol. Chem. **41:**1807–1809. http://dx.doi.org/10.1271/bbb1961.41.1807.
4. **Shima S, Sakai H.** 1981. Poly-L-lysine produced by Streptomyces. Part III. Chemical studies. Agric. Biol. Chem. **45:**2503–2508.
5. **Shima S, Fukuhara Y, Sakai H.** 1982. Inactivation of bacteriophages by ε-poly-L-lysine produced by Streptomyces. Agric. Biol. Chem. **46:**1917–1919.
6. **Takehara M, Hirohara H.** 2010. Occurrence and biosynthesis of poly-epsilon-L-lysine in microorganisms, p 1–22. *In* Hamano Y (ed), Amino-acid homopolymers occurring in nature. Microbiology monographs, vol 15. Springer, New York, NY.
7. **Hirohara H, Takehara M, Saimura M, Ikezaki A, Miyamoto M.** 2006. Biosynthesis of poly(ε-L-lysine)s in two newly isolated strains of Streptomyces sp. Appl. Microbiol. Biotechnol. **73:**321–331. http://dx.doi.org/10.1007/s00253-006-0479-2.
8. **Hirohara H, Saimura M, Takehara M, Miyamoto M, Ikezaki A.** 2007. Substantially monodispersed poly(ε-L-lysine)s frequently occurred in newly isolated strains of Streptomyces sp. Appl. Microbiol. Biotechnol. **76:**1009–1016. http://dx.doi.org/10.1007/s00253-007-1082-x.
9. **Shima S, Matsuoka H, Iwamoto T, Sakai H.** 1984. Antimicrobial action of ε-poly-L-lysine. J. Antibiot. (Tokyo) **37:**1449–1455. http://dx.doi.org/10.7164/antibiotics.37.1449.
10. **Yamanaka K, Maruyama C, Takagi H, Hamano Y.** 2008. ε-Poly-L-lysine dispersity is controlled by a highly unusual nonribosomal peptide synthetase. Nat. Chem. Biol. **4:**766–772. http://dx.doi.org/10.1038/nchembio.125.
11. **Hamano Y.** 2010. Biochemistry and enzymology of poly-epsilon-L-lysine biosynthesis, p 23–44. *In* Hamano Y (ed), Amino-acid homopolymers occurring in nature. Microbiology monographs, vol 15. Springer, New York, NY.
12. **Hamano Y, Arai T, Ashiuchi M, Kino K.** 2013. NRPSs and amide ligases producing homopoly(amino acid)s and homooligo(amino acid)s. Nat. Prod. Rep. **30:**1087–1097. http://dx.doi.org/10.1039/c3np70025a.
13. **Marahiel MA, Stachelhaus T, Mootz HD.** 1997. Modular peptide synthetases involved in nonribosomal peptide synthesis. Chem. Rev. **97:**2651–2674. http://dx.doi.org/10.1021/cr960029e.
14. **Schwarzer D, Finking R, Marahiel MA.** 2003. Nonribosomal peptides: from genes to products. Nat. Prod. Rep. **20:**275–287. http://dx.doi.org/10.1039/b111145k.
15. **Mootz HD, Schwarzer D, Marahiel MA.** 2002. Ways of assembling complex natural products on modular nonribosomal peptide synthetases. Chembiochem **3:**490–504. http://dx.doi.org/10.1002/1439-7633(20020603)3:6<490::AID-CBIC490>3.0.CO;2-N.
16. **Yamanaka K, Kito N, Kita A, Imokawa Y, Maruyama C, Utagawa T, Hamano Y.** 2011. Development of a recombinant ε-poly-L-lysine synthetase expression system to perform mutational analysis. J. Biosci. Bioeng. **111:**646–649. http://dx.doi.org/10.1016/j.jbiosc.2011.01.020.
17. **Kito N, Maruyama C, Yamanaka K, Imokawa Y, Utagawa T, Hamano Y.** 2013. Mutational analysis of the three tandem domains of ε-poly-L-lysine synthetase catalyzing the L-lysine polymerization reaction. J. Biosci. Bioeng. **115:**523–526. http://dx.doi.org/10.1016/j.jbiosc.2012.11.020.
18. **Yamanaka K, Kito N, Imokawa Y, Maruyama C, Utagawa T, Hamano Y.** 2010. Mechanism of ε-poly-L-lysine production and accumulation revealed by identification and analysis of an ε-poly-L-lysine-degrading enzyme. Appl. Environ. Microbiol. **76:**5669–5675. http://dx.doi.org/10.1128/AEM.00853-10.
19. **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. http://dx.doi.org/10.1263/jbb.99.636.
20. **Maruyama C, Toyoda J, Kato Y, Izumikawa M, Takagi M, Shin-ya K, Katano KH, Utagawa T, Hamano Y.** 2012. A stand-alone adenylation

domain forms amide bonds in streptothricin biosynthesis. Nat. Chem. Biol. **8:**791–797. http://dx.doi.org/10.1038/nchembio.1040.

21. **Sambrook J, Russell DW.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
22. **Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA.** 2000. Practical *Streptomyces* genetics. John Innes Foundation, Norwich, United Kingdom.
23. **Itzhaki RF.** 1972. Colorimetric method for estimating polylysine and polyarginine. Anal. Biochem. **50:**569–574. http://dx.doi.org/10.1016/0003-2697(72)90067-X.
24. **Katano H, Yoneoka T, Kito N, Maruyama C, Hamano Y.** 2012. Separation and purification of ε-poly-L-lysine from the culture broth based on precipitation with the tetraphenylborate anion. Anal. Sci. **28:**1153–1157. http://dx.doi.org/10.2116/analsci.28.1153.
25. **Hirokawa T, Boon-Chieng S, Mitaku S.** 1998. SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics **14:**378–379. http://dx.doi.org/10.1093/bioinformatics/14.4.378.
26. **Nishikawa M, Ogawa K.** 2006. Inhibition of epsilon-poly-L-lysine biosynthesis in *Streptomycetaceae* bacteria by short-chain polyols. Appl. Environ. Microbiol. **72:**2306–2312. http://dx.doi.org/10.1128/AEM.72.4.2306-2312.2006.