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MINI-REVIEW

Recent advances in the biotechnological production of microbial poly(ε-L-lysine) and understanding of its biosynthetic mechanism

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Abstract Poly(ε-L-lysine) (ε-PL) is an unusual biopolymer composed of L-lysine connected between α-carboxyl and ε-amino groups. It has been used as a preservative in food and cosmetics industries, drug carrier in medicines, and gene carrier in gene therapy. Modern biotechnology has significantly improved the synthetic efficiency of this novel homopoly(amino acid) on an industrial scale and has expanded its industrial applications. In the latest years, studies have focused on the biotechnological production and understanding the biosynthetic mechanism of microbial ε-PL. Herein, this review focuses on the current trends and future perspectives of microbial ε-PL. Information on the screening of ε-PL-producing strains, fermentative production of ε-PL, breeding of high-ε-PL-producing strains, genomic data of ε-PL-producing strains, biosynthetic mechanism of microbial ε-PL, and the control of molecular weight of microbial ε-PL is included. This review will contribute to the development of this novel homopoly(amino acid) and serve as a basis of studies on other biopolymers.

Keywords Poly(ε-L-lysine) · Biopolymer · Non-ribosomal peptide synthetase · Fermentation · *Streptomyces*

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Introduction

Homopoly(amino acid)s are linear synthetic polymers consisted by repeating units of unique type of amino acid. Four natural homopoly(amino acids)s secreted by microorganisms have been discovered until now encompassing poly(γ-glutamic acid), poly(ε-L-lysine) (ε-PL), poly(γ-L-diaminobutanoic acid), and poly(L-diaminopropionic acid) (Ivanovics and Bruckner 1937; Shima and Sakai 1977; Takehara et al. 2008; Xia et al. 2013). Among these homopoly(amino acid)s, ε-PL is commonly known because of its widespread applications. Structurally, ε-PL is an unusual natural biopolymer consisting of L-lysine monomers connected between adjacent α-carboxyl and ε-amino groups (Fig. 1). Because of its novel structure, ε-PL exhibits many excellent properties, such as biodegradable, water soluble, thermostable, antibacterial, edible, and non-toxic. Moreover, it also exhibits excellent endotoxin-selective removal and antiobesity properties, improves cell adhesion, inhibits pancreatic lipase activity, and prevents oral bacterial toxin production (Hiraki et al. 2003; Shih et al. 2006). Hence, ε-PL has been extensively investigated in food, agricultural, pharmaceutical, and cosmetic industries. More detailed descriptions of these applications with ε-PL are available elsewhere (Bankar and Singhal 2013; Chheda and Vernekar 2015b; Pandey and Kumar 2014; Shih et al. 2006; Shukla et al. 2012). Among such a vast application, ε-PL is mainly used as a natural food preservative because of its safety, heat stability, biodegradability, and broad-spectrum antimicrobial activity (Shih et al. 2006; Shima et al. 1984). In the 1880s, ε-PL was first approved as a food preservative in Japan. Then, it was introduced into the USA, South Korea, and other countries. In 2014, ε-PL and its hydrochloride

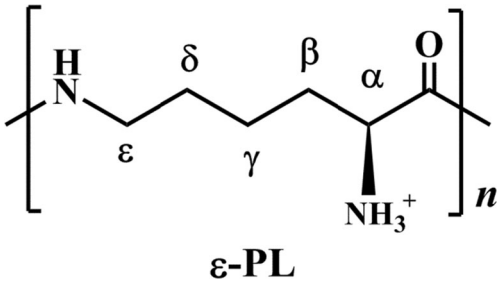

Fig 1 The chemical structure of ε-PL

were also approved by the National Health and Family Planning Commission of China as a food preservative (http://www.nhfpc.gov.cn/sps/s7890/201404/a93467d652c24a75a6de637abde31f30.shtml).

In the industry scale, ε-PL is mainly produced by microorganisms through fermentation. Compared with chemical method, which requires repetitive protection/deprotection reactions and/or many chemical compounds, microorganisms are more practical, efficient, and environment friendly for ε-PL production (Kushwaha et al. 1980; Tao et al. 2015). Microbial ε-PL was first discovered in 1977, when Shima and Sakai screened Dragendorff-positive compounds (Shima and Sakai 1977). Then, the original ε-PL-producing strain was mutated, and some efficient fermentation approaches were employed for high ε-PL productivity. However, no other ε-PL-producing strains had been discovered in a long time because of the lack of effective screening methods appropriate for ε-PL-producing strains. In 2002, a high-efficiency method was developed to screen ε-PL-producing strains by detecting the color zone formed by the secreted ε-PL (Nishikawa and Ogawa 2002). Since then, more and more studies have been performed to exploit the microbial ε-PL, including screening of new ε-PL-producing strains, development of new fermentation processes, control of the molecular weight of ε-PL, and discovery of ε-PL degrading enzymes and synthetase (Hamano et al. 2006; Hirohara et al. 2006, 2007; Nishikawa 2009; Nishikawa and Ogawa 2006; Shih and Shen 2006a, b; Shih et al. 2006; Yamanaka et al. 2008). In recent years, with the development of genetic engineering, bioinformatics, and advanced precision instruments and testing equipment, the biosynthetic mechanism of microbial ε-PL has been investigated more and more. Of course, the efficient production of ε-PL is a constant theme, and several valid fermentation processes were established for ε-PL production in the latest years. This review focuses on the latest progress on the biotechnological production and biosynthetic mechanism of microbial ε-PL. We hope that this review could help researchers understand the current breakthroughs and future trends of this novel biopolymer. Furthermore, studies on the biotechnological production and biosynthetic mechanism of microbial ε-PL could also provide a basis for investigations on other biopolymers.

Screening of ε-PL-producing strains

As mentioned in the "Introduction" section, screening of ε-PL-producing strains is one of the crucial components in the study of microbial ε-PL. To isolate ε-PL-producing strains, one efficient approach was provided using the acidic polymeric dye, Poly R-478, incorporated into agar plates to interact with the ε-PL secreted by target microorganisms. Because ε-PL is a cationic polyamide, and Poly R-478 is an acidic polymeric dye, it could form condensed diffusion zones on agar plates due to the electrostatic interaction. Thus, the target strains were isolated (Nishikawa and Ogawa 2002). With this method, more than 10 ε-PL-producing strains were discovered from soil. Since then, several other efficient ε-PL-producing strains were also screened based on this method or an improved method (Geng et al. 2014; Li et al. 2011; Ouyang et al. 2006). Furthermore, this method could not only be applied for the qualitative screening of ε-PL-producing strains, the density and diameter of the color reaction concentric zone can also reflect the productivity and the molecular weight of the secreted ε-PL. Thus, this novel color reaction method also derived out a series of approaches for the content determination of ε-PL. For instance, Zhang et al. developed an agar diffusion assay method to detect ε-PL in fermentation broths and foods(Zhang et al. 2012). This method was based on the strong electrostatic interaction between ε-PL and methylene blue dye that triggered the appearance of a clear diffusion zone on an agar plate containing methylene blue. The diffusion zone diameter exhibited a highly linear relationship with $\log_{10}$ (ε-PL concentration). In this condition, the concentration of ε-PL can be calculated quickly by measuring the diameter of the diffusion zone.

Another efficient method for screening ε-PL-producing strains is the two-stage culture method of cell growth and ε-PL accumulation (Hirohara et al. 2006; Hirohara et al. 2007). As a result, about 200 ε-PL-producing strains were isolated. With the two main methods mentioned above, several ε-PL-producing strains were screened from the soil, most of them belonging to two groups of the family *Streptomycetaceae* and ergot fungi. In recent years, some *Bacillus* strains were also found to secrete ε-PL (Chheda and Vernekar 2014, 2015a; El-Sersy et al. 2012). These different species may be valuable for understanding the evolution of ε-PL-producing strains and the mechanism of ε-PL biosynthesis. The most popular ε-PL-producing strains are summarized in Table 1, with different molecular weights and productivities of ε-PL. The detailed information regarding these strains is presented in the following sections.

Table 1 ε-PL-producing strains and molecular weight of the secreted ε-PL

Strains	Main carbon and organic nitrogen sources	Key fermentation strategy	ε-PL yield (g/L)	Fermentation time (h)	Molecular weight (kDa)	Main references
S. albulus S410	Glucose + yeast extract	A two stage pH control strategy	48.3	192	3.2–4.5	Kahar et al. (2001)
	Glucose + yeast extract	Airlift bioreactor application	30	168	3.2–4.5	(Kahar et al. (2002)
S. albulus IFO 14147	Glucose + yeast extract	Medium optimization	8.13	72	3.2–4.5	(Shih and Shen (2006a)
	Glucose + yeast extract	pH control and feeding strategy	5.2	252	3.2–4.5	Shih and Shen (2006b)
	Culture filtrate from levan fermentation	Sequential production of levan and ε-PL	4.37	72	NM	Shih et al. (2011)
Kitasatospora sp. MY5-36	Glucose + yeast extract	Cell immobilization	34.1	99	About 5.05	Zhang et al. (2010)
Streptomyces. sp. GIM8	Glucose + yeast extract	Resin-based in situ product removal strategy	23.4	200	3.5–4.5	Liu et al. (2011)
S. albulus TUST2	Glucose + yeast extract	Substrate feeding strategy	20	96	1–4.5	Shiru et al. (2009)
Streptomyces sp. M-Z 18	Glycerol + beef extract	pH control strategy	30.1	174	About 4.21	Chen et al. (2011a)
	Glycerol + Glucose + beef extract	Using glucose and glycerol as a mixed carbon source	35.1	174	About 4.21	Chen et al. (2012)
	Glycerol + fish meal + corn steep liquor	Acidic pH shock strategy	54.7	192	About 4.21	Ren et al. (2015b)
	Glycerol + fish meal + corn steep liquor	Acidic pH shock strategy + talc microparticles addition	62.36	192	About 4.21	Ren et al. (2015c)
S. albulus PD-1	Glucose + yeast extract	Oxygen-vector addition	30.8	168	3.5–4.5	Xu et al. (2015a)
	Glucose + yeast extract	*Vitreoscilla* hemoglobin expression	34.2	168	3.5–4.5	Xu et al. (2015b)
	Hydrolyzed cane molasses + hydrolyzed waste biomass	Waste feedstock application	20.6	168	3.5–4.5	Xia et al. (2014)
S. noursei NRRL 5126	Glycerol + proteose peptone	DO control strategy	1.99	120	<10	Bankar and Singhal (2011)
Bacillus subtilis sp.	Glucose + yeast extract	Medium optimization	0.0763	16	NM	El-Sersy et al. (2012)
Bacillus cereus sp.	Glucose + yeast extract	Glucose + metabolic precursors feeding	0.565	96	NM	Chheda and Vernekar (2015a)
S. griseofuscus H1	Glucose + yeast extract	pH control + fed-batch fermentation strategy	7.5	120	NM	Li et al. (2011)

NM not mentioned in the article

Fermentative production of ε-PL

As mentioned above, ε-PL has been widely applied in various industries. To meet the rapidly increasing demands for ε-PL, its commercial production is indispensable. Since the screening of the first ε-PL-producing strain, several fermentation strategies have been persistently pursued for high ε-PL productivity. The most classic fermentation strategy for ε-PL production is the two-phase pH control approach, and the ε-PL production titer reported with this approach was 48.3 g/L by *Streptomyces albulus* S410 (Kahar et al. 2001). Surprisingly, this work was from 15 years ago, which might suggest that enhancing ε-PL at the current level is very difficult because of the toxicity of ε-PL on its own producing strain and the limited metabolic ability of the producers. Just until recently, the yield of ε-PL titer made a breakthrough and surpassed that of Kahar's research with a pH shock strategy application (Ren et al. 2015b, c). In general, the research for fermentative production of ε-PL is mainly divided into three parts, namely, pH control strategy, dissolved oxygen control strategy, and waste feedstock application. However, this does not mean that other approaches, including the optimization of medium components and the application of new bioreactors, are not necessary; each approach has its own strength that complement the production of ε-PL, which should also be actively pursued to enrich the options for the efficient production of ε-PL. The global optimization of these bioprocess and bioreactor variables will significantly contribute to maximizing ε-PL production and minimizing the overall operation costs.

Fermentative production of ε-PL with pH control strategy

In the submerged fermentation of ε-PL, the pH of culture broth was found to decrease continuously from the initial value (about 6.5–7.0) to about 3.0, accompanied by ε-PL accumulation. However, when the pH of the culture broth was maintained at 5.0 or 6.0, no ε-PL was detected (Shima and Sakai 1981). Further research suggested that the optimum pH for ε-PL accumulation was 4.0 and for cell growth was 6.0. Based on these results, Kahar et al. improved the overall efficiency of ε-PL production from 5.7 to 40.3 g/L using *S. albulus* S410 with two distinct phases; in the first phase, the pH of the culture broth was maintained above 5.0 for cell growth, whereas the pH was maintained at pH 4.0 for ε-PL accumulation in the second phase (Kahar et al. 2001). Recently, Ren et al. modified this classic pH control strategy by integrating it with an acidic pH shock phase. The modified fermentation process can be divided into three phases, namely, pre-acid-shock adaption at pH 5.0, acidic pH shock at pH 3.0, and pH restoration to 4.0 for ε-PL production. Ultimately, the maximum ε-PL titer in the culture broth reached 54.70 g/L. Compared with the classic two-phase pH control strategy by Kahar et al., the metabolic activity of the producing strain was severely inhibited in the pH-shock phase, which induced the overproduction of regulatory, biosynthetic, and stress-response genes. Thus, cell growth and ε-PL production were significantly enhanced, when pH was shifted back to 4.0 (Ren et al. 2015b).

With the aforementioned information, it can be concluded that pH is a crucial parameter for ε-PL biosynthesis. No matter which strategy was carried out, the pH for ε-PL accumulation was always strictly controlled at about 4.0. At first, researchers assumed that ε-PL accumulation in pH 4.0 is attributed to the inhibition of the ε-PL-degrading enzyme (Pld) in the acidic condition. To clarify whether the inhibition of Pld is the ultimate reason for ε-PL accumulation, two *pld* genes were knocked out from the ε-PL-producing strain. However, knocking the *pld* genes out still did not lead to ε-PL accumulation in the neutral pH condition. Further investigations revealed that acidic pH conditions in culture broth are essential for the intracellular ATP accumulation, rather than the inhibition of Plds alone. As mentioned, intracellular ATP is not only directly involved in the assembly of ε-PL by activating L-lysine to L-lysyl-O-AMP, but high ATP levels are crucial for the transcription of the ε-PL synthetase (Pls) gene (Yamanaka et al. 2008, 2010).

Fermentative production of ε-PL with dissolved oxygen control strategy

Apart from pH, dissolved oxygen (DO) level in the culture broth is another key parameter in ε-PL production. Based on the aforementioned information, most ε-PL-producing strains belong to the family *Streptomycetaceae*. Cell growth and ε-PL biosynthesis all need large amounts of ATP, which is mainly generated through aerobic respiration. This indicates that high DO levels in the culture broth would be beneficial for both cell growth and ε-PL biosynthesis. In this regard, Bankar and Singhal systematically evaluated the fermentation kinetics of *Streptomyces noursei* NRRL5126 under different aeration and agitation conditions, and a DO control strategy for ε-PL production was developed. In this condition, a constant DO level at 40 % in cell growth phase and 20 % in ε-PL production phase effectively increased the ε-PL yield titer and biomass to 1.99 and 20.73 g/L (Bankar and Singhal 2011). However, the biomasses of many other ε-PL-producing strains are higher than that of *S. noursei* NRRL5126 (Ren et al. 2015a; Xia et al. 2013). Because of the intertwined hyphae, high cell density, and high molecular weight of ε-PL, the culture broth becomes viscous, and the oxygen transfer efficiency is low. Therefore, it was proved difficult to improve the DO level simply by improving the agitation and aeration during these fermentation processes. Furthermore, the increase of agitation could also lead to undesired effects on mycelial morphology, product formation, and production yield because of the accompanied high shear stress. On this occasion, Xu et al.

attempted to improve the oxygen supply for *S. albulus* PD-1 using two strategies. In the first case, oxygen-vectors were added to the culture broth. The addition of 0.5 % *n*-dodecane in culture broth can efficiently maintain DO levels with >32 % saturation, as opposed to 23.8 % in the ε-PL production stage. Consequently, the ε-PL titer yield was improved from 23.4 to 30.8 g/L (Xu et al. 2015a). In another case, the capability of binding oxygen for *S. albulus* PD-1 was enhanced by introducing *Vitreoscilla* hemoglobin gene into its chromosome (Xu et al. 2015b). With this application, the recombinant strain produced more than 50.7 % ε-PL compared with the wild-type strain.

The determination of intracellular ATP content indicated that more ATP was generated in *S. albulus* PD-1, when abundant oxygen was supplied. It also reflected the importance of ATP in ε-PL biosynthesis from another aspect. Actually, a powerful ATP supply was detected from all the popular ε-PL-producing strains investigated. As an extension of those studies, Yoshimura et al. took full advantage of this excellent characteristic of ε-PL-producing strains and focused on their heterologous hyaluronic acid production, which resulted in the efficient production of hyaluronic acid with high molecular weight (Yoshimura et al. 2015). Probably, ε-PL-producing strains (of course, the *pls* should be deleted) could be superior hosts for heterologous production of a wide variety of bioproducts in the future, as their biosynthesis pathways require amounts of ATP in vivo.

Fermentative production of ε-PL with waste feedstock

In the investigation of ε-PL production, some researchers tried to produce ε-PL using waste feedstock, which not only reduced the cost of industrial ε-PL production but also converted the obsolete resources to this novel value-added compound. Considering the prices and dosages of the medium components, researchers found that some waste feedstock could substitute the carbon and organic nitrogen sources commonly used in ε-PL production. In fact, the most common medium for ε-PL production is medium 3G (M3G), which comprises 50 g/L glucose, 5 g/L yeast extract, 10 g/L $(NH_4)_2SO_4$, 1.36 g/L KH_2PO_4, 0.8 g/L K_2HPO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.04 g/L $ZnSO_4{\cdot}7H_2O$, and 0.03 g/L $FeSO_4{\cdot}7H_2O$. It is obvious that glucose occupies a large portion of the medium component. Thus, some researchers attempted to use alternative carbon resources including glycerol and cane molasses to produce ε-PL, especially high volumes of crude glycerol that have been manufactured as the associated waste product of biodiesel production in recent years (Posada et al. 2012). The most successful application of glycerol in ε-PL production was conducted by the group of Mao, who first optimized the medium for ε-PL production with glycerol as carbon source. Then, a series improvement was conducted based on this medium. Ultimately, ε-PL production was improved to 62.36 g/L in a 5 L bioreactor after 192 h of cultivation from the original 2.27 g/L in flasks (Chen et al. 2011a, b; Ren et al. 2015a, b, c; Sun et al. 2015; Zeng et al. 2014). In addition to *Streptomyces* sp. M-Z18, *S. noursei* NRRL 5126 and *Streptomyces lydicus* USE-11 also used glycerol as the main carbon resource (Bankar and Singhal 2010; Hirohara et al. 2007). In another study, Xia et al. attempted to use hydrolyzed cane molasses to produce ε-PL. After optimizing the initial total sugar concentration, 20.6-g/L ε-PL was obtained in a 1-t fermenter. In this case, the by-product of the sugar industry was successfully transformed to ε-PL (Xia et al. 2014).

The most expensive component of M3G is the organic nitrogen (yeast extract). Hence, alternative nitrogen sources were investigated to replace the yeast extract in M3G. Commonly, large amounts of ε-PL-producing strains were cultivated for ε-PL production and disposing these biomasses became a challenge. Thus, reusing the hydrolysate of the ε-PL-producing strain itself as the renewable and self-cycling nitrogen source was favored in ε-PL production (Xia et al. 2014). In a similar study, the filtrate of the culture broth for levan production was utilized for ε-PL production, and a sequential fermentation process for levan and ε-PL production was established (Shih et al. 2011). This process is ingenious and environmentally friendly, because it takes full advantage of the nutrient elements in the waste culture filtrate generated during levan production. Fish meal coupled with corn steep liquor was also used as an alternative organic nitrogen source for industrial ε-PL production. As described, this strategy can produce a net profit increase of 9057 USD in a 1-t fermenter (Ren et al. 2015a). In general, recycling these agro-industrial by-products is favorable for the large-scale production of ε-PL, especially that the feedstock has become increasingly expensive in recent years.

Some other typical strategies for the production of ε-PL

In addition to fermentative strategies mentioned above, other effective measures have also been taken to improve the production of ε-PL, which plays an important complementary role in biotechnological ε-PL production. In general, these strategies could be divided into two parts, namely, medium optimization and application of new bioreactors. Components in the medium could affect the primary and secondary metabolism of the microorganisms, which is ultimately reflected in cell growth and product synthesis. Medium optimization is a conventional approach for biotechnology, and when a new target microorganism was screened, researchers attempt to find a suitable medium for it. As early as 2006, Shih et al. developed an appropriate medium for *S. albulus* IFO 14147 by surface response methodology. As a result, the ε-PL secreted by *S. albulus* IFO 14147 was increased about ninefold (Shih and Shen 2006b). Similarly, Bankar and Singhal

optimized medium components using Placket-Burman design method for ε-PL production with *S. noursei* NRRL 5126 (Bankar and Singhal 2010). Recently, Chheda and Vernekar used orthogonal array method to determine the optimum medium composition for a novel ε-PL producer *Bacillus cereus*, and the ε-PL titer increased significantly from 36.29 to 83.49 mg/L (Chheda and Vernekar 2014). Thus, although medium optimization method may involve with a heavy workload, it was found to be quite effective for ε-PL production.

The type of bioreactor used is another important factor during the fermentation process. The most commonly used bioreactor in ε-PL production is the conventional bioreactor with mechanical agitation and regular aeration. However, researchers have not given up on designing new bioreactors for ε-PL production. As early as 2002, an airlift bioreactor was evaluated for ε-PL production, which promised low production cost, energy consumption, and downstream processing (Kahar et al. 2002). Because ε-PL is a cationic polymer, the anionic resin could be used to absorb the accumulated ε-PL. With this application, the ultimate ε-PL titer yield was significantly increased by overcoming the feedback inhibition and toxic effects from the accumulated ε-PL (Liu et al. 2011). Cell immobilization is another useful tool for ε-PL production. Zhang et al. used loofah sponge as a green immobilization material for ε-PL production. In this case, ε-PL titer yield and productivity reached 34.1 g/L and 9.3 g/(L·d), respectively. Moreover, the immobilized cells can be used up to five times, and almost no lag phase was observed except in the first batch, which greatly shortened the fermentation period (Zhang et al. 2010). This novel immobilization device has already been applied in Shineking Biological Technology Company. It is noteworthy that all ε-PL productions reported by now were performed through submerged fermentation. The loofah sponge used in the cell immobilization study was something like the solid substrate in solid-state fermentation. Maybe, solid-state fermentation will be applied to ε-PL in the near future.

Breeding of high-producing strains for ε-PL

In the previous sections, the isolation of ε-PL-producing strains and the fermentative production of ε-PL were discussed. All biotechnologists know that an excellent producer is the primary determinant for an industrial-scale production. For ε-PL production, several high yield mutants have been obtained by using conventional and modern breeding techniques, including chemical mutagenesis, genome shuffling, and genetic engineering. For this system to work, Hiraki et al. screened an L-lysine analog, S-(2-aminoethyl)-L-cysteine, and glycine-resistant mutant, producing four times more ε-PL (Hiraki et al. 1998). Genome shuffling technique was carried out on *Streptomyces graminearus* LS-B1, and one glucose tolerant strain was obtained, which enhanced the initial glucose concentration in culture broth to 85 g/L and the final ε-PL titer yield to 13.5 g/L (Li et al. 2012). In connection with this study, a combined genome shuffling and interspecific hybridization method among *Streptomyces* was used. Five ε-PL-producing strains were carried out for genome shuffling, and the ε-PL titer yield of a mutant strain achieved 24.5 g/L, which was >63 % higher than the wild types (Li et al. 2013).

In recent years, rational genetic engineering has improved the overproduction of various primary and secondary metabolites. Some genetic alterations have also been performed to generate highly productive ε-PL-producing strains. However, the lack of well-developed gene delivery systems and the complex physiology of most ε-PL-producing organisms limit the speed of ε-PL-overproducing-strains development. For *S. albulus* IFO14147 and *S. albulus* PD-1, the only two ε-PL-producing strains with their own gene delivery systems, some genetic modifications have already been introduced into their chromosomes with interesting results. Hamano et al. created an ε-PL-high-producing strain with modified Ask, rAsk (M68V), whose feedback inhibition regulation from L-lysine was completely removed, and thus more precursors were provided for ε-PL overproduction (Hamano et al. 2007). This is similar to screening ε-PL-producing mutants with S-(2-aminoethyl)-L-cysteine and glycine-resistant mutants. Recently, a gene delivery system was constructed for *S. albulus* PD-1 through conjugational transfer, which resulted in the integration of *Vitreoscilla* hemoglobin expression cassette into the chromosome of *S. albulus* PD-1 to solve the oxygen limitation during fermentation (Xu et al. 2015b). From these results, it seems intriguing to improve the ε-PL synthetic ability of the producing strains by gene manipulation. However, not all genetic manipulations achieved significant effects. Yamanaka et al. knocked out the Plds of *S. albulus* CRM001, but no discernible ε-PL increase was observed (Yamanaka et al. 2010). Thus, the identification and systematic understanding of the regulatory and biosynthetic systems of ε-PL are important to improve ε-PL production by genetic engineering in further studies. Recently, high-throughput techniques, their resulting omics data, and in silico models provided massive amounts of information to understand the biosynthesis of ε-PL and to construct ε-PL-overproducing strains.

Genome sequence reports of ε-PL-producing strains

Biopolymer synthesis is a complicated procedure that involves cell growth, precursor synthesis, energy provision, redox equilibrium, and transportation of substrates and products. All these factors are essential for ε-PL

biosynthesis, but most of them are vague. Currently, cheap gene sequencing has led to the generation of numerous genome sequences for *Streptomyces* and related species, which can help us understand the gene information of the ε-PL metabolic pathway. By now, a total of five ε-PL-producing strains have been sequenced (Table 2). Among the five bacteria, four of them are *S. albulus*, and their genomes exhibit high similarity with each other. As reported, *Streptomyces* have some of the large genomes within the range of 8.7 to 11.9 Mb and GC contents within the range from 69 to 76 % (Harrison and Studholme 2014; Zhou et al. 2012). It shows that the ε-PL-producing strains possess the basic features of *Streptomyces*. For example, comparative genomic analysis indicated that the chromosomal region position from 2.0 to 8.0 Mb of *S. albulus* ZPM was conserved and exhibited significant synteny among *Streptomyces* species. However, further studies revealed that these ε-PL-producing strains also exhibited special characteristics. For instance, analyses using bioinformatics revealed 44 gene clusters for secondary metabolites in *S. albulus* ZPM, which is almost twice the 25 gene clusters of *Streptomyces coelicolor* A3 (2) (Wang et al. 2015).

Based on genomic data, most genes encoding proteins for metabolic, genetic, and environmental information processing in the ε-PL-producing strains were successfully annotated. From associated genomic information, the L-lysine in these ε-PL-producing strains is biosynthesized from L-aspartate and terminated by ε-PL synthetase. Although L-lysine is the precursor for ε-PL biosynthesis, further investigations on the ε-PL-producing strain genome reveal that ε-PL biosynthesis is not only related to the biosynthesis and assemble of L-lysine but is also associated with multiple cellular processes. For example, carbohydrate transport and metabolism are essential components of central metabolism. It not only produces precursor metabolites for ε-PL biosynthesis but also generates reducing equivalents and energy. The determination of intracellular metabolite concentration and enzyme activities has proven this point (Li et al. 2012; Xu et al. 2015a). Because the biosynthetic pathway of ε-PL is strictly regulated through complex global regulatory systems, genomic information will enable more sophisticated projects on metabolic analysis and reconstruction, which can further improve the synthetic efficiency of ε-PL.

In addition to information on ε-PL biosynthesis, other gene clusters could be also mined from the genomic data of ε-PL-producing strains. For instance, nine polyketide synthases (PKS) clusters, six non-ribosomal peptide synthetase (NRPS) clusters, and five hybrid PKS-NRPS clusters were found in *S. albulus* ZPM (Wang et al. 2015). Many of these gene clusters may be silenced, but some of them are expressed at high levels and are not clearly defined. In our previous study, we found another homopoly(amino acid)s, poly(L-diaminopropionic acid), which was co-produced with ε-PL by *S. albulus* PD-1. With the help of the *S. albulus* PD-1 genome, the biosynthesis pathway of poly(L-diaminopropionic acid) was elucidated, which provided a solid basis for the co-production of these two novel homopoly(amino acid)s (Xia et al. 2013; Xu et al. 2015c, d). In conclusion, the genomic information will be a powerful tool for the analysis of other metabolic products and identification of the candidate enzymes in ε-PL-producing strains. With the help of these genomic data, a minimal genome would probably be constructed for potential ε-PL production in the near future.

Biosynthetic mechanism of microbial ε-PL

After the discovery of microbial ε-PL, researchers have tried to understand its synthetic mechanism. Shima et al. reported on the incorporation of L-[^{14}C] lysine into ε-PL using the washed mycelium of *S. albulus* no. 346, and the results indicated that L-lysine is the precursor of ε-PL (Shima et al. 1983). However, the mechanism behind the assembly of the L-lysine monomers into a polymer was unknown. After a long quiescent period, Kawai et al. (2003) achieved ε-PL biosynthesis in a cell-free system. The synthesizing activity was detected in the membrane fraction, which depended heavily on ATP and was not influenced by ribonuclease, kanamycin, or chloramphenicol. These results indicated that the biosynthesis of ε-PL is most likely catalyzed by NRPSs existing in the membrane (Kawai et al. 2003). Five years later, Yamanaka et al. successfully purified and characterized the native form of Pls from the

Table 2 The genomic data of ε-PL-producing strains

Species and strain	GenBank accession numbers	Genome size (Mb)	G + C contents (%)	References
S. albulus CCRC 11814	AROY00000000	9.43	72.2	Dodd et al. (2013)
S. albulus PD-1	AXDB02000000	9.21	72.3	Xu et al. (2014)
S. albulus NK660	CP007574	9.14	72.3	Gu et al. (2014)
S. albulus ZPM	CP006871	9.56	72.2	Wang et al. (2015)
Kitasatospora sp. MY5-36	NZ_LFVW01000001	9.14	73.5	Unpublished

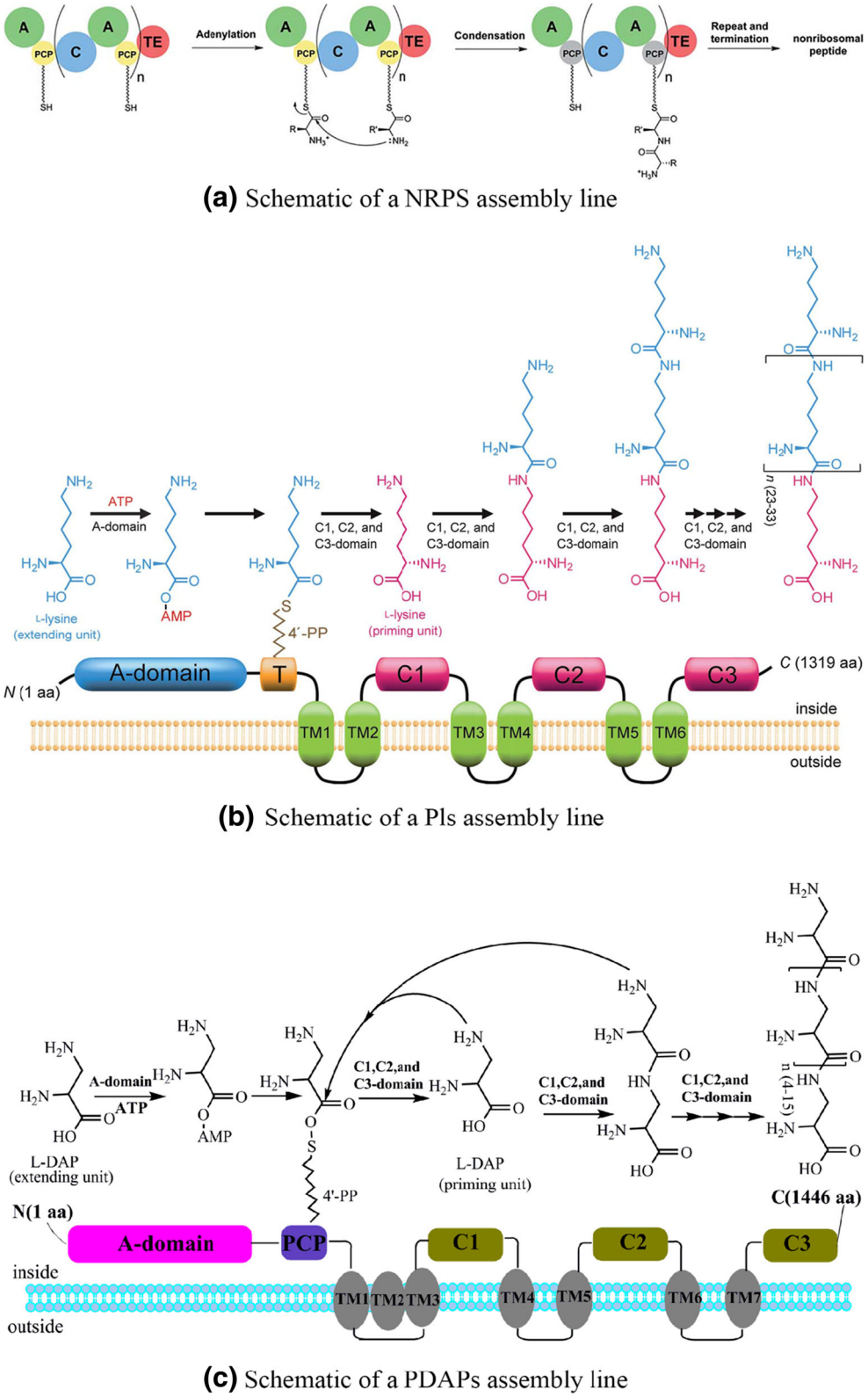

Fig 2 Domain architectures and the catalytic mechanisms of traditioanl NPRSs, Pls, and PDAPs. This figure was made with some modifications using the figure in the previous studies (Condurso and Bruner 2012; Hamano et al. 2013; Xu et al. 2015d)

membrane fraction, and the encoding gene of Pls was also located in the chromosome of *S. albulus* NBRC14147(Yamanaka et al. 2008). By analyzing the structure of Pls, this enzyme was identified to be a highly unusual NRPS. For traditional NRPSs, four domains are ubiquitous: adenylation (A) domains for the selection and activation of the candidate substrates; peptidyl carrier protein (PCP) or thiolation (T) domains for the propagation of the growing peptide chain; condensation (C) domains for

the condensation of the amino acids (the A-PCP/T-C elements are ubiquitous in NRPSs and essential for peptide elongation); and a thioesterase (TE) domain catalyze the release of the production in the end of assembly line (Li et al. 2014; Strieker et al. 2010) (Fig. 2a). In contrast, Pls was found to be a single-module NRPS with a classic A-domain and a classic T-domain of NRPSs. However, no traditional C-domain and TE-domain were found; instead, six transmembrane (TM) domains surrounding three tandem soluble domains (C1, C2, and C3 domains) in the C-terminal were observed (Fig. 2b). Based on the structure of Pls, its catalytic mechanism was predicated and was experimental confirmed: the catalysis of Pls was initiated by the A- and T-domains with the adenylation and transfer of an incoming L-lysine monomer as an extending unit; then, the C-terminal tandem domains catalyzed the peptide bond formation between the extending unit and a freely diffusible L-lysine (priming unit), producing a L-lysine dimer; also, the dimer was used as a freely acceptor for the next polymerization reaction, and a series of ε-PL with multitude chain lengths was generated because this catalytic cycle has no predetermined endpoint. (Yamanaka et al. 2008). The recent research showed that the synthetase of poly(L-diaminopropionic acid) (PDAPs) shares similar conserved domains with Pls (Xu et al. 2015d) (Fig. 2c). Maybe, this novel single-module NRPS structure is a common characteristic of these homopoly(amino acid)s synthetases.

With continuous research, the success of the heterologous expression of Pls opened the possibility to study Pls in other model hosts, which might be suitable for protein engineering and for elucidating its catalytic mechanism (Yamanaka et al. 2011; Geng et al. 2014). Initially, the *pls* was expressed via the control of a constitutive promoter; however, neither Pls expression nor ε-PL production was observed. Ultimately, the native promoter was shown to be effective for Pls expression in either *S. albulus* or *Streptomyces lividans*. In a recent study, a his-tagged Pls was expressed in soluble form and purified by Ni-affinity chromatography (Yamanaka et al. 2011). With the help of this powerful expression system, mutation analysis was carried out for Pls from *S. albulus* NBRC14147 and some key functional sites were screened, such as the conserved block sequence RxLGxxxG in C1, C2, and C3 domains and Ser553 in the T-domain (Kito et al. 2013). Finally, despite the improved comprehension on the structure and functional mechanism of Pls, the current understanding of ε-PL biosynthesis is still limited. For instance, why is the molecular weight of ε-PL unfixed? and why do some Pls synthesize ε-PL with 25–35 residues and some with 15–20 or less? Perhaps the crystal structure of Pls will give the final answer in the future.

In addition to Pls, Pld activity was detected in both ε-PL-producing and ε-PL-tolerant microorganisms. Pld plays an essential role in ε-PL degradation and in self-protection of ε-PL producers. In 2002, Kito et al. first purified and characterized Pld from an ε-PL-producing strain (Kito et al. 2002). This kind of Pld was found to be a membrane protein with exo-type activity, and the enzyme releases N-terminal L-lysine of ε-PL one by one. Moreover, this kind of Pld also can act on various peptides possessing L-lysine residues at the N-terminus except for ε-PL and was classified as an aminopeptidase. Then, the *pld* gene was inactivated from *S. albulus*, but the result strain still exhibited ε-PL-degrading ability, which indicated the existence of other Plds (Hamano et al. 2006). In 2010, when researchers analyzed a 33 kb nucleotide sequence containing the *pls*, they found a metalloproteinase gene adjacent to *pls*. This gene was certified to encode another Pld (PldII). Further examination revealed that Plds exhibited the highest activity at around pH 7.0, whereas the enzyme activities under acidic conditions were considerably low. Thus, it is easy to assume that the accumulation of ε-PL at acidic fermentation conditions (pH 4.0) was regarded as the way to inhibit Pld. However, a knockout mutant of both *pldI* and *pldII* (the mutant strain lost the ε-PL-degrading activity) still could not secrete ε-PL in neutral pH conditions. Therefore, the hypothesis stating that the ε-PL accumulation at acidic conditions was due to the inhibition of Pld activity was inaccurate. In another study, the transcription analysis of *pls* and a kinetic study of Pls stated above further suggested that Pls catalytic function was regulated by the intracellular ATP. The acidic pH conditions during ε-PL fermentation, other than the inhibition of the Plds, were also considered indispensable for the accumulation of intracellular ATP (Yamanaka et al. 2010).

Molecular weight of microbial ε-PL

By now, more and more biopolymers have been discovered from microorganisms, and many of them have been widely applied in all walks of life. Generally, these biopolymers showed a remarkable dispersion in molecular weight. Molecular weight is one of the most important features of these biopolymers, and the function of a given biopolymer is primarily dependent on its molecular weight. For example, Shih pointed out that poly(γ-L-glutamic acid) with 10^2 kDa to 8×10^3 kDa is an excellent viscosity-adding agent, but this property also limits its applicability; in contrast, poly(γ-L-glutamic acid) with 30–50 kDa could be applied as drug delivery for tumor therapy (Bajaj and Singhal 2011; Shih and Van 2001). Hyaluronic acid with a high molecular weight could maintain cell integrity and water content in the extracellular matrix; in contrast, the degraded fragments, oligosaccharide hyaluronic acid, could induce receptor-mediated intracellular signaling (Badle et al. 2014; Jia et al. 2013; Stern et al. 2006). Like the other biopolymers mentioned above, molecular weight is also an important feature for ε-PL. The antimicrobial activity of ε-PL has been reported to be dependent on its molecular size. The ε-PL with chain length less than nine L-

lysine residues exhibited negligible antimicrobial activity. However, when the L-lysine residues were increased to 10, ε-PL strongly inhibited microbial growth. When L-lysine residues exceeded 15, no apparent growth in antibacterial activity was observed. Currently, the commonly used ε-PL commonly ranges from 3.0 to 4.5 kDa (corresponding to 25–35 L-lysine residues) and was manufactured using *Streptomyces*. These ε-PL-producing strains could produce 35–60-g/L ε-PL after 168–192-h fermentation. However, very few ε-PL with other molecular weights appears in the market because of the poor yield of the producing strain. Although several ε-PL-producing strains have been screened to secrete ε-PL with low molecular weight, the ε-PL production is still very low compared with the aforementioned strains. For instance, only 4.5-g/L ε-PL (consisting of 5–20 residues) was obtained with *Streptomyces aureofaciens* USE-82 (Hirohara et al. 2006).

Over a long period in the past, researchers believed that the molecular size of ε-PL was strongly associated with Pld. They also expected increased productivity and/or changes in the molecular weight of the ε-PL produced through the inactivation of the *pld* genes. However, no alterations in the productivity or molecular weight of ε-PL were observed when both *pldI* and *pldII* were inactivated. The in vivo experiments revealed that the molecular weight of ε-PL was decided by the Pls instead of Pld. In a recent study, a *pls* gene mutation library was constructed, and seven mutation variants were found to produce ε-PL with shorter chains. Further analysis found that these variants have one or more mutations in linker regions connecting the TM domains, such as the Try646 and Leu883 amino acid in Pls (accession: BAG68864). For example, when the 646th amino acid was changed from tryptophan to serine, the ε-PL chain length changed from "25–35" to "9–23" (Hamano et al. 2014). Although some key amino acids were founded in Pls, the processes that control the chain size of ε-PL during the polymerization reaction are still not fully understood. As said before, the crystal structure of Pls may give the final answer in the future.

Apart from the Pls and the producing strain itself, the molecular weight of ε-PL is also influenced by the medium components. Additional short-chain polyols or modified cyclodextrins in the culture broth were also observed to regulate the chain length of ε-PL. Nishikawa and Ogawa found that ε-PL elongation was inhibited by the polyols via esterification; the ε-PL esters were formed during L-lysine polymerization, and the number of L-lysine residues of ε-PL-polyol esters decreased with increasing polyol concentration (Nishikawa and Ogawa 2006). Thus, it is feasible to control the number of L-lysine residues in ε-PL by regulating the polyol concentration in the culture broth. Moreover, the addition of chemically modified cyclodextrins (such as sulfated cyclodextrin) in the culture broth could also shorten the polymer length of ε-PL from 3.5–4.5 kDa to less than 2.5 kDa (Nishikawa 2009). Maybe, ε-PL could be biosynthesized efficiently with a customized molecular weight in the near future by more precise molecular weight control system.

Conclusion

With the insights mentioned in this paper, some interesting and practical issues related to microbial ε-PL should be investigated in the future. For example, the screening and construction of more efficient ε-PL-producing strains, continuous and in-depth studies on the catalysis mechanism of Pls, precise control of the molecular weight of the secreted ε-PL, and other fermentation forms, such as solid-state fermentation, can be applied to produce ε-PL. It is anticipated that the ε-PL could be biosynthesized efficiently with a customized molecular weight in the future.

Compliance with ethical standards

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

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

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