Inhibition of Epsilon-Poly-l-Lysine Biosynthesis in Streptomycetaceae Bacteria by Short-Chain Polyols

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Antimicrobial epsilon-poly-l-lysine (ePL) is secreted by Streptomycetaceae bacteria, and the mechanism of ePL biosynthesis remains to be elucidated. We previously reported that an unknown ePL derivative accumulates in the culture medium of ePL-producing bacteria when glycerol is added to the culture medium (Nishikawa and Ogawa, Appl. Environ. Microbiol. 68:3575–3581, 2002). In this study, by using matrix-assisted laser desorption ionization—time of flight mass spectrometry and nuclear magnetic resonance, we identified the unknown derivative as the ester formed between the hydroxyl group of a glycerol molecule and the terminal carboxyl group of an ePL molecule. When a short-chain aliphatic polyol, such as ethylene glycol, propanediol, or butanediol, was added instead of glycerol, a corresponding ePL-polyl monooester accumulated in the culture medium of ePL-producing bacteria. ePL esterification was accompanied by ePL synthesis in intact cells and a cell-free system, but no esterification of exogenous ePL was observed. ePL-polyl esters were formed during lysine polymerization. The number of lysine residues of ePL-polyl esters decreased with increasing polyol concentration. Taken together, these results indicate that ePL synthesis is inhibited by polyols via esterification and that ePL elongation occurs via the incorporation of lysine monomers into the carboxyl terminus of ePL.

*e-Poly-l-lysine (ePL) is a strong basic poly(amino acid) secreted by various Streptomycetaceae bacteria and a few filamentous fungi (16, 20–23). This linear polymer is constructed from the amide bonds between ɛ-amino and α-carboxyl groups (5, 21). Industrially, ePL is produced by fermentation. Because ePL is harmless to humans (7), its antimicrobial activity has been utilized in food preservation. Furthermore, the ability of ePL to suppress fat absorption in the small intestine (10, 24) is of interest for use in health foods. However, natural long-chain ePL tastes bitter. Thus, control of the chain length is important for improving the taste of ePL for consumption (23). Efforts to alter ePL chain length in the industrially established strain Streptomycyes albulus 346 (20) have not been successful. This is probably because of the present poor understanding of ePL biosynthesis.

There are two possible mechanisms underlying the activation of amino acids for peptide biosynthesis: adenylation (AMP forming) by nonribosomal peptide synthetases (NRPSs) and phosphorylation (ADP forming) by amide ligases. NRPSs usually produce fixed-length peptides except for streptothricin (nourseothricin), which has a variable-length poly(L-lysine) chain (9, 25). In contrast, ADP-forming amide ligase produces variable-length peptides, such as poly-γ-DL-glutamate (2), in addition to dipeptides, such as D-alanyl-D-alanine and γ-L-glutamyl-L-cysteine (14, 15). In contrast to poly-γ-glutamate produced by Bacillus species (1), ePL has a relatively low molecular mass and a narrow molecular mass distribution. For example, ePL produced by S. albulus 346 (=IFO14147) consists of approximately 25 to 35 lysine monomer units (molecular mass range, 3,200 to 4,500 Da), and the ratio of mass-average molecular mass to number-average molecular mass is 1.14 (13). Recently, Kawai et al. (8) reported that ePL synthetic activity occurs on the cell membrane of ePL-producing bacteria and suggested that adenylated L-lysine is an activated reaction intermediate in ePL synthesis on the basis of l-lysine-dependent AMP formation. Although ePL has a chain length distribution similar to that of poly-γ-DL-glutamate, it appears that the synthetase for ePL is similar to an NRPS rather than to an ADP-forming amide ligase, considering adenylated L-lysine as a reaction intermediate. However, details concerning ePL synthesis remain obscure.

We previously developed a sensitive and convenient method of screening for microorganisms that secrete acidic or basic substances using electrically charged dyes (16). This method allows easy detection of ePL-producing variants based on their ePL dispersion in agar plates. We isolated a Streptomycetes bacterium, Kitasatospora kifunense MN-1, that produces an unknown, chemically modified ePL derivative in the presence of glycerol in the culture medium (16). Preliminary mass spectrometric analysis predicted that the derivative is formed at one of the termini of the linear ePL molecule. To our knowledge, there have been no reports of a derivative of a terminus of poly(amide acids), including poly-γ-glutamate in Bacillus subtilis and related species, cyanophycin (a cyanobacterial multi-γ-arginyl-poly-L-aspartic acid), and phytocelatin. The three poly(amide acids) are produced by ADP-forming amide ligases. In this study, we identified the unknown ePL derivative produced by K. kifunense MN-1 and determined how it is produced, which led to an outline of the mechanism of ePL biosynthesis.

MATERIALS AND METHODS

Bacterial strains. K. kifunense MN-1 was isolated from forest soil (16). S. albulus IFO14147 was obtained from the Institute for Fermentation, Osaka (Osaka, Japan).
Production of ePL derivatives. MN-1, which produces relatively small ePLs (8- to 17-mers), was cultured in a jar fermentor containing 600 ml of Shima-Sakai (SS) liquid medium (21). Incubation was carried out at 28°C for 6 days. The pH of the culture decreased from 6.8 to 4.2 spontaneously as the bacterium grew and was kept constant at 4.2 by adding a sodium hydroxide solution. After the first 24 h of incubation, 10 g of glycerol and 2 g of (NH₄)₂SO₄ were added to the culture medium every 24 h. As an alternative method of producing an ePL derivative, MN1 was cultured on synthetic glycerol agar plates (16), and the extracellular material secreted was extracted from agar pieces in an aqueous solution of hydrogen chloride.

Assay for esterification of exogenous ePL. For cell-free ePL synthesis, a detergent-solubilized membrane fraction of IFO14147 was prepared by the method of Kawai et al. (8), with slight modifications. 1,3-Butanediol was used instead of glycerol, and SM-2 Bio-Beads (Bio-Rad Labs, California) were used to remove the detergent Brij 58. Either synthetic N²-fluorescin isothiocyanate (FITC)-labeled octameric ε-poly-L-[¹³C₆, ¹⁴N₂]lysine (Peptide Institute, Inc.) at a concentration of 12.5 µM was added to the reaction mixture containing the cell extract, 100 mM Tris-HCl (pH 8.0), 8% 1,3-butanediol, 5 mM MgCl₂, 5 mM dithiothreitol, 5 mM ATP-2Na, and 5 mM N-[(¹³C₆, ¹⁴N₂)lysine-HCl (minimum, >98 atom%); Isotec, Inc., Miamisburg, OH). After incubation at 28°C for 15 h, trichloroacetic acid was added to stop the reaction. After centrifugation, the supernatant was neutralized and subjected to SPE using SepPak CM. The reaction products were eluted with 100 mM HCl and then purified by SPE using ZipTip µC₁₈ (Millipore, Billerica, MA).

RESULTS

Unknown polymer was ePL-glycerol ester. MALDI-TOF mass spectrometry showed that the molecular masses of ePLs produced by K. kifunense MN-1 during agar plate culture ranged from 1,300 to 2,900 Da at regular intervals of about 128 Da (Fig. 1A). This interval corresponds to the mass of a lysine residue in ePL. This strain also produced unknown polymers whose the masses were distributed at the same intervals as those of the ePLs (Fig. 1A). Thus, the unknown polymers were ePL derivatives. An ePL molecule has one free carboxyl group. To determine whether the terminal carboxyl group of an unknown ePL derivative is free, methyl esterification of a mixture of ePL and the ePL derivative was carried out. This experiment resulted in new mass peaks corresponding to ePL methyl esters (Fig. 1B), but there appeared to be no peaks that originated from methyl esterification of the unknown ePL derivative. These results indicate that the terminal carboxyl group of the unknown ePL derivative was modified, since it was protected against methyl esterification.

To determine the mass increment which resulted from the modification of the newly detected ePL derivative, a single-molecular-mass ePL derivative was subjected to partial acid hydrolysis for the purpose of removing the modifying group.
The ePL derivative used in this experiment was produced by MN-1 during liquid culture. In contrast to the sample obtained from a solid culture of MN-1, which contained mainly the ePL derivative along with a small amount of ePL (Fig. 1A), the sample obtained from the liquid culture of MN-1 contained only the ePL derivative (data not shown). It is probable that the difference was due to the sample extraction step specific to the solid culture. Mass analysis revealed no structural differences in the ePL derivative between the two samples (data not shown). Acid hydrolysis converted the ePL derivative with an $m/z$ of 2,014.2 to a molecule with an $m/z$ of 1,940.4, which corresponded to a pentadecameric ePL (Fig. 2). Thus, the mass increase due to modification was about 74 Da. Taking the mass of water (18 Da) into consideration, the modification was thought to be due to incorporation of a molecule having a molecular mass of about 92 Da (74 Da + 18 Da) into the carboxyl group of ePL.

The formation of ePL derivatives required the presence of glycerol in the culture medium. When 5% D-glucose was added instead of glycerol, no ePL derivatives were found. Because the molecular mass of glycerol is 92.1 Da, glycerol might modify ePL to produce ePL derivatives. $^{13}$C-NMR analysis (Fig. 3) using 1-oleoyl-glycerol as a reference compound confirmed that the unknown polymer is an ester formed between the terminal carboxyl group of ePL and the first hydroxyl group of glycerol, that is, 1-ε-poly(L-lysyl)-glycerol (Fig. 4).

Formation of ePL-polyol ester. Similarly, S. albulus IFO14147 also produced an ePL-glycerol ester. When a short-chain aliphatic diol, such as ethylene glycol, 1,2-propanediol, 1,3-propanediol, 1,2-butanediol, 1,3-butanediol, 1,4-butanediol, 2,3-butanediol, or 1,5-pentanediol, was added to the culture medium

![FIG. 2. Positive-ion MALDI-TOF mass spectra of a single-molecular-mass ePL derivative produced by K. kifunense MN-1 during liquid culture. A single molecule of an ePL derivative with a molecular mass of about 2,014 Da was recovered from a mixture of variable-length ePL derivatives by HPLC. The mass spectra of a single molecule before (A) and after (B) partial acid hydrolysis are shown on the same scale. The peak ($m/z$ 1,940.4) appearing after hydrolysis corresponds to the pentadecameric ePL peak.](image)

![FIG. 3. NMR profile of ePL derivative produced by K. kifunense MN-1 during liquid culture.](image)

![FIG. 4. Chemical structure of ePL-glycerol ester.](image)
FIG. 5. Positive-ion MALDI-TOF mass spectra of various ePL-diol esters produced by S. albulus IFO14147. ePL and its esters were extracted from a liquid culture containing only D-glucose (5%) (A) or D-glucose supplemented with one of the following diols at a concentration of 2.5%: ethylene glycol (B), trimethylene glycol (1,3-propanediol) (C), 1,3-butanediol (D), or pentamethylene glycol (1,5-pentanediol) (E). n, number of lysine residues. The underlined and italicized values are the observed and theoretical values for the difference in molecular mass between unmodified ePL and ePL esters, respectively.

of IFO14147 together with D-glucose, the corresponding ePL-diol ester accumulated in the culture medium. Representative MALDI-TOF mass spectra are shown in Fig. 5. For example, the formation of ePL-ethylene glycol ester was confirmed by an increase of 42.9 in the m/z from m/z 3,990.8 to m/z 4,033.7 (Fig. 5A and B). This increase corresponded closely to the theoretical value (44.05) calculated by subtraction of the molecular mass of water (18.02) from the molecular mass of ethylene glycol (62.07). Addition of a short-chain polyol resulted in a decrease in the length of the ePL-polyol ester. Among the polyols examined, relatively long-chain polyols, such as pentamethylene glycol (Fig. 5E) and octamethylene glycol (data not shown), tended to have strong effects on the decrease in poly-

mer length; however, the formation of ePL-octamethylene glycol ester could not be confirmed because the molecular masses of L-lysine and octamethylene glycol are nearly equal and could not be differentiated by mass spectrometry. An ester was produced in a culture containing meso-erythritol (1,2,3,4-butanetetrol) but not in a culture containing 1,4-cyclohexanediol, myoinositol, or sorbitol (data not shown).

Correlation between esterification and polymerization of ePL. To examine whether ePL esterification is coupled with lysine polymerization, we investigated the time course of ePL and ePL-glycerol ester accumulation in an IFO14147 culture. ePL-glycerol esters started to accumulate during the early stationary phase (between 28 and 32 h after the start of the culture). We did not observe free ePLs (nonesterified ePLs) prior to accumulation of the ePL-glycerol esters or throughout the culture (Fig. 6A to D). Therefore, these results suggest that newly polymerized ePL is immediately esterified. To examine whether ePL esterification is inseparably linked to the polymerization of lysine, we added ePL to a culture synthesizing ePL and determined whether ePL esterification occurred. Exogenous ePL was not esterified (Fig. 6F). Although the ability of ePL to permeate the cell membrane remains obscure, it is clear that an exogenous ePL is not esterified on the cell surface or in the extracellular space while endogenous ePL synthesis is active. Therefore, ePL esterification is likely to be accompanied by ePL synthesis.

To assess the possible esterification of the carboxyl terminus of exogenous ePL regardless of the permeability of ePL, we added ePL to a cell-free lysine-polymerizing system prepared from IFO14147. ePL having a molecular mass between 3.2 and 4.5 kDa, which was biologically synthesized by IFO14147, was used as an exogenous ePL applied to the in vitro ePL-synthesizing system. Unfortunately, it was difficult to discriminate between the newly polymerized ePL and the exogenously added ePL, because the latter was degraded, which resulted in a molecular mass shift from 3.2 to 4.5 kDa to 0.8 to 1.7 kDa (data not shown). Therefore, we added N'-FITC-labeled octameric ePL consisting of L-[13C6, 15N2]-lysine to the cell-free system together with 1,3-butanediol and L-[13C6, 15N2]-lysine. This agent facilitated discrimination between the newly polymerized ePL and the exogenously added ePL (Fig. 7). An ester between 13C- and 15N-labeled ePL and 1,3-butanediol was observed, as was nonesterified 13C- and 15N-labeled ePL (Fig. 7A). On the other hand, N'-FITC-labeled ePL was not esterified (Fig. 7B). The FITC-labeled ePL was degraded to smaller molecules (trimers to hexamers) (Fig. 7B) by an unknown peptidase that was probably different from the aminopeptidase reported previously (11), but it was not elongated to form larger molecules by the uptake of lysine. Since the FITC-labeled ePL oligomer representing exogenous ePLs was not esterified and was not a substrate for lysine polymerization, it is possible that ePL esterification is accompanied by L-lysine polymerization.

When polyols were added to a culture of IFO14147, the number of lysine residues in the ePL ester decreased. In the absence of polyol, nonesterified ePLs ranging from 24- to 35-mers accumulated in the culture (Fig. 8A). In the presence of 1,5-pentamethylene glycol, ePL-1,5-pentamethylene glycol ester accumulated in the culture. The number of lysine residues in the ePL-1,5-pentamethylene glycol ester decreased from 28 to 13 when the 1,5-pentamethylene glycol concentration in-
creased from 0.625 to 2.5% (Fig. 8B to D). Cell growth was not inhibited in the presence of 5% 1,5-pentamethylene glycol, whereas ePL production was completely inhibited (Fig. 8E). Other polyols, such as ethylene glycol, 1,3-propanediol, and 1,3-butanediol, similarly shortened the polymer (Fig. 5B to D). Thus, we concluded that polyols inhibit L-lysine polymerization via esterification.

DISCUSSION

Elucidation of ePL esterification is important for addressing obscure points regarding ePL biosynthesis. Here, we found that ePL esterification was coupled with ePL synthesis, which is basically the polymerization of lysine monomers, and that the number of lysine residues in the ePL ester decreased with increasing concentrations of the polyol added to the culture. These results indicate that ePL synthesis is inhibited by polyols via esterification, and it is likely that the elongation of ePL results from incorporation of lysine monomers into the carboxyl terminus of ePL. Generally, peptide synthesis is catalyzed by either an ADP-forming amide ligase or an AMP-forming NRPS. The former catalyzes condensation of an amino acid into the carboxyl group of a free peptide; the latter catalyzes a series of peptide bond formations between an amino acid and a nascent peptide, both of which are anchored via thioester bonds (12, 19). Cyanophycin synthetase, a member of the ADP-forming amide ligase superfamily, was experimentally shown to condense amino acids using a C-terminus-free short peptide as a primer for peptide elongation (3). In contrast, the inability of ePL to elongate by incorporating t-lysine into a C-terminus-free N\textsuperscript{ε}-FITC-labeled ePL octamer (Fig. 7) is evidence that ePL is synthesized in a “peptide-anchored-to-an-enzyme” manner, similar to the action of NRPSs, and not in an amide ligase-like manner.

Recently, Kawai et al. (8) suggested that adenylated t-lysine is a reaction intermediate in ePL synthesis. Their results support the proposal that a poly(lysyl)-thioester is formed during ePL synthesis using NRPSs as an analogy, because this type of amino acid activation (adenylation) is characteristic of NRPSs. We clarified that ePL synthesis by the cell-free system is inhibited by a sulfhydryl group-blocking agent, N\textsuperscript{-}ethylmaleimide (data not shown). Although this reagent is not specific for the catalytic center of NRPSs, it is possible that the inhibition of ePL synthesis resulted from blocking of the SH groups in active sites. It is also possible that a potential SH group(s) regulating the enzyme activity via conformational change was blocked by N\textsuperscript{-}ethylmaleimide. To identify the inhibition, development of an effective quantitative assay for ePL synthesis is necessary.

If all the available information, including that from a previous report (8), is integrated, ePL synthesis and its inhibition by polyols can be assumed to occur as follows. Lysine polymerization proceeds by iterative reactions between at least two active aminoacyl thioester intermediates in a manner similar to the action of NRPSs. In the initial round, two free lysine residues are loaded onto two free SH groups via lysyl-AMP formation, and then the ε amino group of one lysyl-thioester nucleophilically attacks the carbonyl of another lysyl-thioester to form one lysyl-lysyl-thioester and one free SH group. In the
In the next round, one free lysine is loaded onto the free SH group, and then the ε amino group of the newly formed lysyl-thioester attacks the carbonyl of the lysyl-lysyl-thioester to form one lysyl-lysyl-lysyl-thioester and one free SH group. In this way, lysine monomers are added to the carboxyl terminus of nascent ePL, which remains tethered to the putative ePL synthetase. The two active SH groups do not accept any free ePLs, such as an Nε-FITC ePL octamer. ePL is usually released from the ePL synthetase by hydrolysis when the number of lysine residues is sufficient. In the presence of short-chain aliphatic polyols, the poly(lysyl)-thioester is easily interrupted by polyols, and finally the elongating ePL peptide is released from the active site. However, at present, the detailed mechanism of lysine polymerization and esterification is not understood.

More work needs to be carried out to support the proposed mechanism of ePL synthesis. We are interested in the synthesis of ePL in relation to the synthesis of a novel class of variable-length linear peptides via thioester formation. Currently, only the biosynthesis of the poly-β-lysine chain of streptothricin fits under this classification, although this peptide is not free but is associated with a d-gulosamine core. The gene encoding the β-lysine-adenylating enzyme involved in poly-β-lysine chain assembly is well characterized (4, 6). The deduced amino acid sequence exhibits homology with other NRPS family members. If ePL synthesis is achieved by iterative operation of the putative thiotemplate mechanism in a manner similar to the action of NRPSs, it is the first example of NRPS-like synthesis of a free variable-length linear peptide. Poly(l-arginyl-D-histidine) exhibits variation in peptide length (17), but its biosynthesis mechanism is still unknown. On the basis of the presence of biologically rare D-histidine in this molecule (18), poly(L-arginyl-D-histidine) is a candidate for one of the variable-length peptides synthesized in an NRPS-like manner, such as streptothricin and possibly ePL. NRPSs often convert the chirality of component amino acids during peptide synthesis. To date, no activity that directly converts L-histidine to D-histidine independent of poly(L-arginyl-D-histidine) synthesis has been detected.

Here, we propose a simple method for controlling the number of lysine residues in ePL by regulation of the polyol concentration in the culture medium. ePL-producing bacteria other than S. albulus IFO14147 and K. kifunense MN-1 also produce ePL-polyol esters (data not shown). These ePL esters are generally converted to free ePL under alkaline conditions. This method has potential to change the chemical properties of ePL, such as polarity, and to add new functions to ePL by ester formation with appropriate polyols.

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