Appl Microbiol Biotechnol (2006) 73:321–331
DOI 10.1007/s00253-006-0479-2

Biosynthesis of poly(ε-L-lysine)s in two newly isolated strains of *Streptomyces* sp.

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Received: 10 March 2006 / Revised: 16 April 2006 / Accepted: 18 April 2006 / Published online: 7 September 2006

Abstract The biosynthesis of poly(ε-L-lysine) (ε-PL) in the two newly isolated strains of *Streptomyces lydicus* USE-11 (USE-11) and *Streptomyces* sp. USE-51 (USE-51) was studied by a newly developed two-stage culture method of cell growth at pH 6.8 and ε-PL production at pH 4.5. USE-11 synthesized ε-PL consisting of about 28 residues at a high production level, whereas USE-51 did the polymer with 15 ones at a low level. The secreted ε-PLs in culture media were digested in a neutral pH range with a peptide hydrolase(s) produced by the ε-PL producers. The optimum production levels were presumed to be dependent upon the inherent ε-PL synthesis machinery of each producer. The production in USE-51 was sharply dependent upon cell density as was often observed in the production of antibiotics, whereas that in USE-11 was scarcely affected by the density. The SO_4^{2-} was found to be essential for the ε-PL production in both strains. This might suggest the involvement of a thiol group in the polymerization reactions including the activation of L-lysine. This study indicates that USE-11 is a most suitable strain for the exploration of the ε-PL biosynthesis at the molecular level as well as for the technical applications.

Electronic supplementary material Electronic supplementary material is available if you access this article at http://dx.doi.org/10.1007/s00253-006-0479-2 and is accesible for authorized users.

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Introduction

Poly(ε-L-lysine) (ε-PL) is an L-lysine homopolymer biosynthesized nonribosomally and has a unique structure linking ε-amino and α-carboxylic acid functional groups (Fig. 1). The polymer with 25–30 residues was discovered as a secreted product from a strain of *Streptomyces albulus* No. 346 (now available as *S. albulus* IFO 14147) in culture filtrates (Shima and Sakai 1977). The compound is biodegradable, water-soluble and independent of oil-based resources, and has a variety of biological and chemical functionalities such as antimicrobial activity (Shima et al. 1984; Hiraki 2000), antiphage action (Shima et al. 1982), IgM and interferon-β production stimulatory activities (Maeda-Yamamoto et al. 1995), effectiveness as drug delivery carriers (Shih et al. 2004), and superabsorbent, endotoxin-selective removal abilities when in hydrogels (Kunioka and Choi 1995; Hirayama et al. 1999; Kunioka 2004). All these specific properties were studied using ε-PL samples from *S. albulus* No. 346. Conventional mutation breeding (Hiraki et al. 1998) and efficient fermentation methods (Kahar et al. 2001, 2002) of the strain were also investigated to enhance ε-PL production. Because of its low environmental impact due to biodegradability, ε-PL-degrading enzymes and its correlation with ε-PL-producing activities have been studied in several microorganisms (Kito et al. 2002a,b, 2003). The polymer is nonmutagenic and practically nontoxic in acute, subchronic, and chronic feeding studies in rats (Hiraki et al. 2003). The ε-PL is manufactured in a real commercial scale by a fermentation process using a mutant of the *S. albulus* strain and is used as a preservative for Japanese foods in many countries, including Japan, Korea, and the United States (Hiraki 2000; Yoshida et al. 2003; Yoshida and Nagasawa 2003; Hamano et al. 2005).

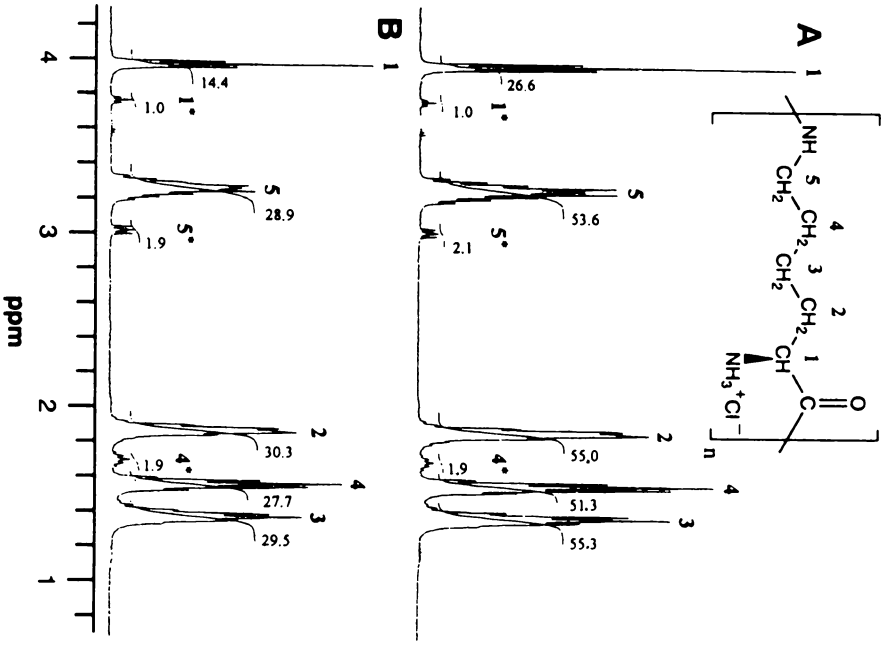

Fig. 1 Chemical structure of poly(ε-L-lysine) hydrochloride (ε-PL7HCl) and ^{1}H NMR spectra of polymers produced in the strain USE-11 (USE-11) (**a**) and in the strain USE-51 (USE-51) (**b**). Resonances labeled with *asterisks* denote the terminal methine and methylene protons of the polymers

In spite that the polymer is scientifically so interesting and practically so useful, reports on ε-PL are very limited so far both in quantity and in aspects examined compared to those on poly(γ-glutamic acid) (γ-PGA), the other naturally occurring secretory amino acid homopolymer (Ashiuchi and Misono 2002, 2003; Oppermann-Sanio and Steinbüchel 2002), or cyanophycin (CGP), the storage amino acid polymer which accumulates inside producing cells (Oppermann-Sanio and Steinbüchel 2002, 2003). Substantially, no intensive study was reported on the biosynthesis of ε-PL yet. These might be mainly attributed to the fact that ever since the first discovery of the *S. albulus* strain, no microorganisms producing ε-PL have been isolated until recently when a new screening method using an acidic dye, Poly R-478, succeeded in finding several ε-PL-producing species of *Streptomyces* and ergot fungi (Nishikawa and Ogawa 2002).

γ-PGA was discovered 40 years before ε-PL as a cell capsule substance (Ivánovics and Erdös 1937). Many experiments performed over the following years clarified that the compound was synthesized by various members of the genus *Bacillus* (Kunioka 1997; Ashiuchi and Misono 2003) and also by other organisms (Weber 1990; Hezayen et al. 2000). The molecular structure including L/D isomer ratio, nutritional requirements, and biosynthetic pathways were studied in a variety of producers (Kunioka 1997 and references therein). Recently, synthetic systems have been investigated at the molecular level (Ashiuchi et al. 1999, 2004; Urushibata et al. 2002). CGP, discovered in the 19th century, has been well studied on the biosynthesis molecular biologically (Oppermann-Sanio and Steinbüchel 2002, 2003).

Because of great interest in practical use and in basic research, the investigation of the biosynthesis of ε-PL has been strongly desired by obtaining a strain suitable for such a study. Under these circumstances, we have performed a screening study for a producer worth examining its ε-PL production in detail and report in this study the characterization of the ε-PL biosynthesis by two newly isolated remarkable strains. This is the first report on the control of ε-PL production and might stimulate further studies to explore a variety of aspects of ε-PL such as the isolation of ε-PL-synthesizing enzymes, the cloning of their encoding genes, or the biosynthesis mechanism. Radioisotopic detection of ε-PL synthesized with a cell-free system from the strain No. 346 was reported recently (Kawai et al. 2003).

Materials and methods

Chemicals, enzymes, and bacterial strain

The purified ε-PL7HCl prepared with a mutant of *S. albulus* No. 346 was provided by Chisso (Tokyo, Japan). Chemically synthesized poly(α-L-lysine) hydrobromide (α-PL7HBr) was purchased from Wako Pure Chemicals (Osaka, Japan). Protease A from *Aspergillus oryzae* and trypsin (Type IX from porcine pancreas) were obtained from Amano Enzyme

(Nagoya, Japan) and Sigma (St. Louis, MO, USA), respectively. Yeast extract, malt extract, cycloheximide, nystatin, and all other chemicals used (analytical grade) were from commercial sources. *S. albulus* IFO 14147 was obtained from the Institute for Fermentation, Osaka, Japan.

Screening procedures

By focusing screening targets on the group of actinomycetes, approximately 1,000 actinomycete strains were isolated by the soil dilution plate method. Soil samples (ca. 1 g) collected from Kansai districts, Japan were suspended with 10 ml of 0.85 % NaCl solutions and incubated with shaking (200 rpm and 2.4 cm rotation diam) for 10 min and without shaking for 30 min at 30 °C. Supernatants were diluted 100 to 1,000 times adding the saline and 0.1 ml of the resulting solutions spread onto glycerol-Czapek plates [1.5 % agar (Difco, Detroit, MI, USA)] containing cycloheximide and nystatin (each at 50 mg l^{-1}) as antifungal reagents. Plates were incubated for 3 to 7 days at 30 °C, and actinomycetes colonies were selected by their appearances and subcultured onto yeast extract–malt extract agar plates (ISP medium 2 agar). Then, a newly developed two-stage culture method was applied to soil actinomycetes for obtaining strains secreting ε-PL. A two-stage culture was proposed by Shima et al. (1983) from the observation that there was a discrepancy between the optimum pH of ε-PL accumulation and cell growth in *S. albulus* No. 346.

Firstly, a loopful of each colony was inoculated into a 10-ml test tube containing 3 ml growth culture medium (20 g glycerol, 5 g yeast extract, 0.5 g $MgSO_4 7 7H_2O$, 0.13 g KH_2PO_4, and 0.14 g Na_2HPO_4 in 1 l, pH 6.8) with a stainless steel coil [5 (diam) by 10 mm] providing dispersed growth and cultured for 20–48 h at 30 °C with shaking (200 rpm and 2.4 cm in rotation diam) (cell growth culture).

Secondly, the mycelium collected by centrifugation (1,700×*g*, 10 min, 4 °C) was washed with 3 ml of the saline and resuspended with the same volume of production medium (220 mM glycerol, 95 mM citrate, 76 mM $(NH_4)_2SO_4$, and 11 mM L-lysine, pH 4.5 with NaOH) and cultured for up to 7 days at 30 °C (ε-PL production culture).

Identification of strains USE-11 and USE-51

ε-PL-producing strains USE-11 and USE-51 were thoroughly purified from contaminated bacteria and fungi by streak plates (ISP 2 agar) and examined with a Philips XL20 scanning electron microscope (Philips Electron Optics, Eindhoven, The Netherlands). Chemical analyses of amino acids in cell walls were performed by the methods of Staneck and Roberts (1974). The amplified 16S rDNA sequencing method was not sufficient for a species identification in the genus *Streptomyces* in contrary to many other bacteria. Thus, taxonomic identification of the strains was deputed to the NCIMB (Aberdeen, Scotland). Identification was carried out according to the probability matrix described by Williams et al. (1983) as well as the Bergey's Manual of Determinative Bacteriology (Holt et al. 1994) and other literatures (cited in these two references). USE-11 and USE-51 were deposited in the collection of the International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (Tsukuba, Japan) as strains FERM P-16810 and FERM P-17223, respectively.

Culture conditions for ε-PL production

The two-stage culture method described above was applied for ε-PL production throughout this study as a standard except for the following experimental conditions. After a 25 % glycerol stocked strain at −80 °C was subcultured with ISP medium 2 for 2 weeks, both cell growth and ε-PL production cultures were conducted with 100 ml culture media containing several stainless steel coils in 500-ml Sakaguchi flasks with rotational shaking (180 rpm and 7 cm diam) for 12–72 h (the first stage) and up to 20 days (the second stage). The production culture broths were analyzed for factors affecting the polymer production. Typically, 1 ml of the culture broth was pipetted out at given time intervals. A half of the sample was used for the determination of the concentration of ε-PL with an anionic dye, Methyl Orange (MeO), and the rest was used in pH and protein content measurements. All the experiments were performed more than once, and most were repeated more than twice.

Purification of MeO precipitating substance (ε-PL7HCl)

The culture filtrate was adjusted to pH 2.5 with an HCl solution and saturated with a methanol/acetone (3:1) mixture 30 to 67 % for strain USE-11 and 40 to 67 % for USE-51. After standing overnight at 4 °C, the resulting precipitate was dissolved in deionized water and purified by ion-exchange chromatography on a TSKgel CM-5PW column (Tosoh, Tokyo, Japan) equilibrated with 50 mM sodium phosphate buffer (pH 7.6). Elution was performed with a linear gradient of NaCl in the buffer. The MeO precipitating fractions were collected, concentrated, and desalted by ultrafiltration [YM 1 (Millipore, Bedford, MA, USA)]. Partially purified ε-PLs were applied to the same column again and decolorized with active carbon. Finally, thoroughly purified ε-PL7HCl was obtained as a white powder by the addition of acetone (85 % saturation) at an acidic pH.

Identification of the purified polymer

The amino acid composition of the purified polymer was analyzed after hydrolysis (6 M HCl, 110 °C, for 24 h) by the

precolumn derivatization method with 4-(dimethylamino) azobenzene-4′-sulfonyl chloride as recommended by the supplier (JASCO, Tokyo, Japan). The L/D isomer ratio of the residue was determined by a high-performance liquid chromatography (HPLC) on a Crownpak CR (+) column (Daicel, Tokyo, Japan) in a perchloric acid solution at pH 1.4 and 10 °C. The specific rotation of the purified polymer was measured using a DIP-1000 polarimeter (JASCO) with a microcuvette [3 (diam) by 100 mm].

The purified polymer (1.0 mg ml^{-1}) was treated with 1.0 mg ml^{-1} Protease A in 50 mM phosphate buffer (pH 7.0), or with 5.0 μg ml^{-1} trypsin in 50 mM Tris–HCl (pH 8.0) and 2.0 mM $CaCl_2$ at 37 °C for 90 min. The concentration of the unhydrolyzed polymer was measured with the MeO precipitating method. The purified ε-PL7HCl sample from a mutant of *S. albulus* No. 346 and α-PL7HBr were treated under the same conditions and used as references.

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a JEOL JNM-LA400 FT NMR spectrometer at 400 MHz. The polymers for measurement were prepared as ca. 1.3 % solutions in D_2O containing a small amount of deuterated TMSP [3-(trimethylsilyl) propionic acid sodium salt], and chemical shifts were measured at 25 °C with 5-mm diam tubes.

Quantitative determination of ε-PL and other organic compounds

The concentration of ε-PL in various solutions was determined on the basis of the method of Itzhaki (1972) with some modifications. A half milliliter of sample solutions was added to 2 ml MeO solutions (1 mM MeO and 50 mM sodium phosphate, pH 7.0) and vortexed. After standing for 30 min at room temperature, mixtures were centrifuged at 1,700×*g*, and the resulting supernatants were diluted tenfold. Absorbances of resulting dilutions were measured at 470 nm. The purified ε-PL7HCl sample was used as a control. The concentration of the polymer was shown as ε-PL in the free amine form throughout this study.

Organic compounds such as glycerol or citrate in culture broths were measured using an HPLC system with a refractive index or a UV detector (JASCO), respectively, equipped with an Aminex HPX-87H organic acids analysis column (Bio–Rad, Hercules, CA, USA). Glycerol concentrations were also determined with enzymatic assays using a Food Analysis kit (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined by the method of Lowry with bovine serum albumin as a standard.

Electrophoretic methods

Purified ε-PL7HCl was analyzed by tricine–sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 15 % polyacrylamide separating gel and a 3 % stacking gel at pH 8.8 (Schägger and von Jagow 1987). Running buffer comprised of 0.1 M Tris, 0.1 M tricine, and 0.1 % SDS. An SDS-PAGE polypeptide marker kit (Bio–Rad) containing triosephosphate isomerase (26.6 kDa), myoglobin (17.0 kDa), α-lactalbumin (14.4 kDa), aprotinin (6.5 kDa), insulin B-chain oxidized (3.5 kDa), and bacitracin (1.4 kDa) was used for estimating molecular weights. Bands were stained with Coomassie Brilliant Blue G-250.

Results

Identification of producer strains

The screening study has given us two strains USE-11 and USE-51 capable of excreting great and reasonable amounts of MeO precipitating substances in the culture media, respectively. LL-Diaminopimelic acid in the cell wall confirmed that the two isolates belonged to the genus of *Streptomyces*. From morphological, physiological, and chemotaxonomical results, the strain USE-11 was identified with the *S. lydicus* in cluster group 29 and designated *S. lydicus* USE-11 (USE-11). The strain USE-51, on the other hand, was assigned to a new *Streptomyces* species and designated *Streptomyces* sp. USE-51 (USE-51).

Molecular structures of produced polycations

Our main target was ε-PL, which would be secreted in the culture medium and precipitated with MeO. The precipitation would, however, occur for any substance having more than three cationic sites in the molecule as we confirmed that MeO formed insoluble complexes with such polycations. Hence, we first determined chemical structures of excreted polycations from USE-11 and USE-51. Both compounds gave positive results for the biuret reaction. Amino acid analyses revealed both peptidic compounds were composed of only lysine. Peptides consisted of only L-isomer in an HPLC analysis using a chiral column (data not shown on these results). The treatments by Protease A or trypsin showed that the poly(L-lysine)s and the purified ε-PL7HCl sample were hydrolyzed by the former enzyme but were not by the latter which digested the α-PL7HBr (see Table ESM-1).

Chemical structure of ε-PL7HCl and ^{1}H NMR spectra together with peak assignments including those of the terminal methine and methylene groups for the poly (L-lysine)7HCls from USE-11 and USE-51 are shown in Fig. 1, which indicated that both polymers were ε-PL7HCls having linear forms in their molecular structures. From the signal area ratio of internal to terminal groups, the degrees of polymerization of ε-PL7HCls from USE-11 and USE-51 were estimated to be about 28 and 15, which corresponded

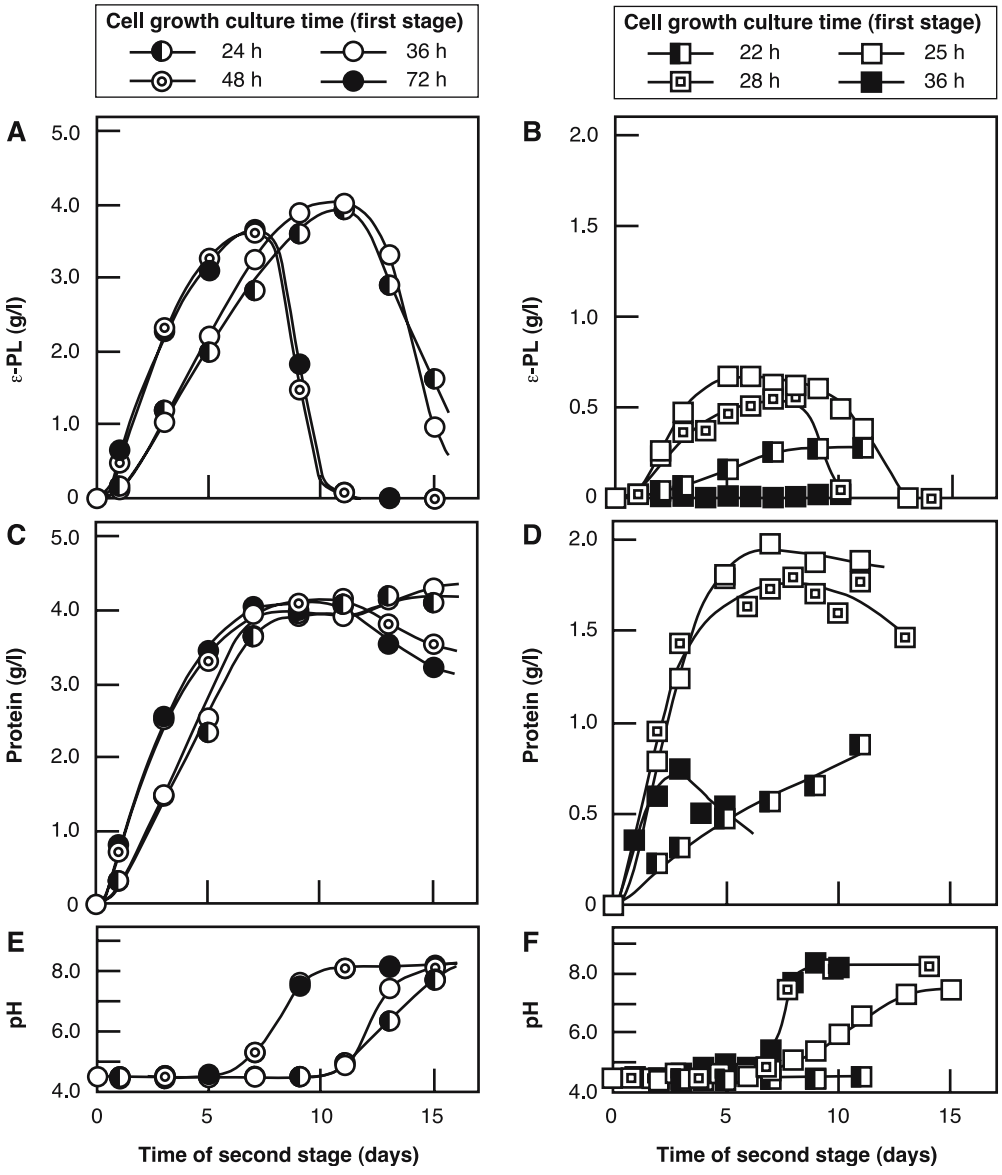

Fig. 2 Time courses of ε-PL production (**a**, **b**), protein excreted (**c**, **d**), and pH values of culture medium (**e**, **f**) in USE-11 (*left-hand side*) and USE-51 (*right-hand side*) cells growth-cultured for a variety of times. The standard production culture medium (see "Materials and methods") was used in all runs

to molecular weight being $4.6*10^3$ and $2.5*10^3$, respectively. SDS-PAGE analysis of the polymers with standard polypeptides indicated relative molecular mass estimates of ε-PLs from USE-11 and USE-51 to be 4.5 and 3 kDa, respectively, and these molecular masses were not changed by fermentation conditions such as growth culture time or culture medium during production. Values for USE-11 were in agreement with those reported previously for ε-PL7HCl from *S. albulus* No. 346 (Shima and Sakai 1981), whereas those for USE-51 were rather large when compared to those recently reported (Nishikawa and Ogawa 2002).

The polymers from USE-11 and USE-51 exhibited $[\alpha]_D^{25}+40.4°$(6 M HCl, c=2.1) and $+40.9°$(6 M HCl, c=1.3), respectively, which are in good agreement with the previously reported value $\left\{[\alpha]_D^{25}+39.9°(6\text{ M HCl},c=1)\right\}$ (Shima and Sakai 1981).

Cell growth culture time and ε-PL production

Time courses of ε-PLs and total proteins excreted into culture broths, together with pH values of media, were studied with cells growth-cultured for a variety of times (Fig. 2). ε-PL production by the two strains exhibited the common phenomenon of the reduction of polymer levels to zero after reaching optimums. Such disappearance corresponded well with pH enhancements from pH 4.5 to pH 8 in culture media (Fig. 2a,e as well as Fig. 2b,f). The rate and the optimum level of ε-PL production in USE-51 were

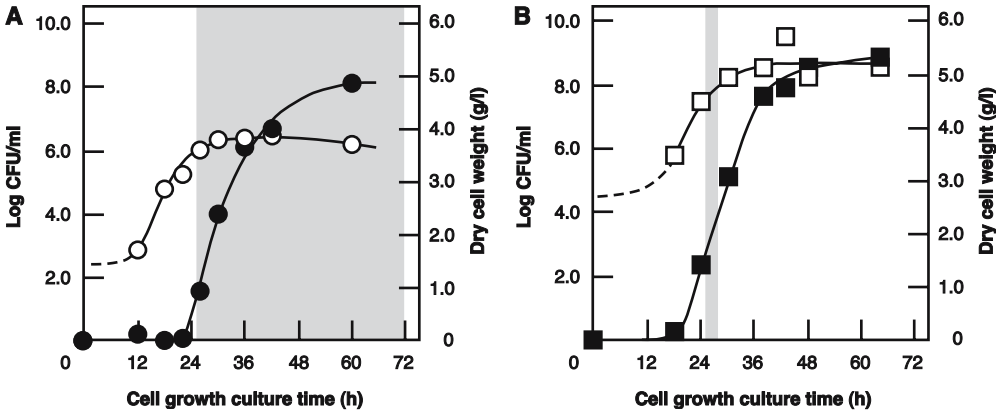

Fig. 3 Viable counts and cell weight of USE-11 (**a**) and USE-51 (**b**) during cell growth cultures. Viable counts (○ and □) were estimated by dilution plating (CFU per milliliter), and cell weight (● and ■) was determined by gram of dried cells per liter of culture. Cell growth culture times for the optimum production of ε-PL are indicated as a *shadow zone*. USE-11 cells form firm flocs in growth media so that viable counts are underestimated

sharply dependent upon cells which were growth-cultured. The 25 h growth-cultured cells gave the highest production rate and level among all the cells cultured from 22 to 36 h. The low level of ε-PL was produced at a slow rate with the 22 h cultured cells, and no polymer was detected with the 36 h cultured cells (Fig. 2b).

In contrast, the production of USE-11 was hardly affected by cell growth culture time. Production levels and rates with long growth cultures (48–72 h) were only slightly lower and faster than those with 24 or 36 h cultured cells. The USE-11 strain produced much more ε-PL than USE-51 (Fig. 2a,b) and more than *S. albulus* IFO 14147 (~2.8 g l^{-1}) under the present experimental conditions. As noted from Fig. 2a,c, more than 80 % of secreted proteins could be ε-PL at the optimal level of the production in USE-11, although proteins and ε-PL were measured with different methods.

Cell growth culture time normally reflects the phase of cell proliferation. We thus monitored time courses for the CFU number and the dry cell weights for the two strains (Fig. 3). The suitable growth phase for ε-PL production read from Fig. 2a,b, illustrated with a shadow, was quite narrow for USE-51 from late-exponential to very early-stationary phases (Fig. 3b). USE-11, in contrast, yielded the polymer throughout a wide range from mid-exponential to late-stationary phase (Fig. 3a).

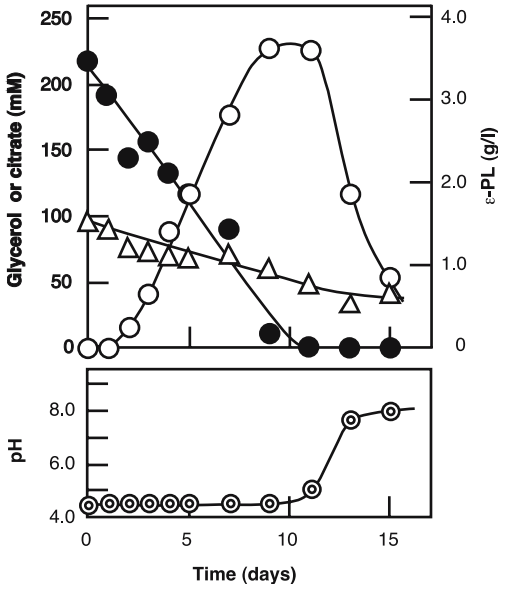

Fig. 4 Concentration of glycerol (●) and citrate (△), and pH value of culture medium (◎) during ε-PL production (○) in USE-11 cells growth-cultured for 33 h. Culture medium initially consisted of 76 mM $(NH_4)_2SO_4$ and 11 mM L-lysine·HCl besides glycerol and citrate

Effect of feeding and consumption of glycerol on ε-PL production

The pH enhancement accompanied by the disappearance of ε-PL was associated with the exhaustion of glycerol instead of citrate having a buffer action. The phenomena were illustrated with USE-11 (Fig. 4). Similar curves were also obtained with USE-51. Such a pH enhancement might reflect the occurrence of ammonia in the culture broth after the consumption of glycerol. Indeed, the broth occasionally smelled slightly of ammonia in the late stage of the fermentation. It was confirmed with USE-51 that successive feeding of glycerol to compensate for the consumption not only maintains the production level but also the pH value (see Fig. ESM-1). The maintenance of production level and pH value by the feeding was also observed with USE-11.

Effect of organic acid on ε-PL synthesis

Effects of organic acids in the citric acid cycle (TCA-C) were examined on the ε-PL production with USE-51 (Fig. 5). Among the acids, citrate facilitated the production and yielded the highest level of the polymer, whereas

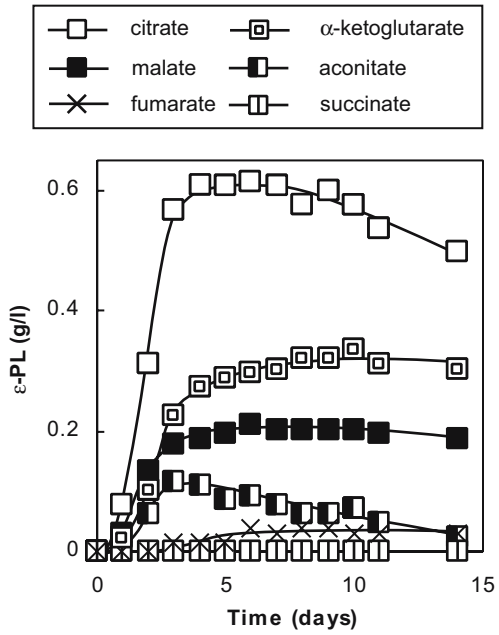

Fig. 5 Effect of organic acids in the citric acid cycle on ε-PL production at pH 4.5 in USE-51 cells growth-cultured for 25 h. The production culture medium consisted of 35 mM of each organic acid, 220 mM glycerol, and 76 mM $(NH_4)_2SO_4$. L-Lysine7HCl was not added to the medium to explicitly observe the effect of the acid

succinate completely inhibited the polymer production. Other organic acids in the cycle were in-between (Fig. 5). A few acids not belonging to the cycle such as acetate and lactate strongly prohibited both strains from producing the polymer even in the presence of the equal mole fractions of citrate in the medium. Very similar tendencies were observed in USE-11.

The effects of citrate concentration were examined on the production, which showed a facilitating effect at adequate concentrations (e.g., 30 or 50 mM) together with a slight retardation at a rather high concentration of 95 mM (see Fig. ESM-2). Very similar results were also observed with USE-51.

Nitrogen source and sulfate

Ammonium sulfate was found to yield the prominently best results among various nitrogen sources such as NH_4Cl, NH_4NO_3, $NaNO_3$, urea, casamino acid, polypeptone, or yeast extract for USE-11. This indicates that SO_4^{2-} is crucial, and the NH_4^+ form is the most effective nitrogen source for the ε-PL production. In fact, the addition of a rather low concentration of 25 mM Na_2SO_4 to a medium containing 100 mM NH_4Cl exhibited the highest production (Fig. 6). It was, however, noticed that 50–76 mM $(NH_4)_2SO_4$ gave better results than 25 mM in the production (Fig. 6). Small amounts of the polymer were produced without the addition of Na_2SO_4 or $(NH_4)_2SO_4$

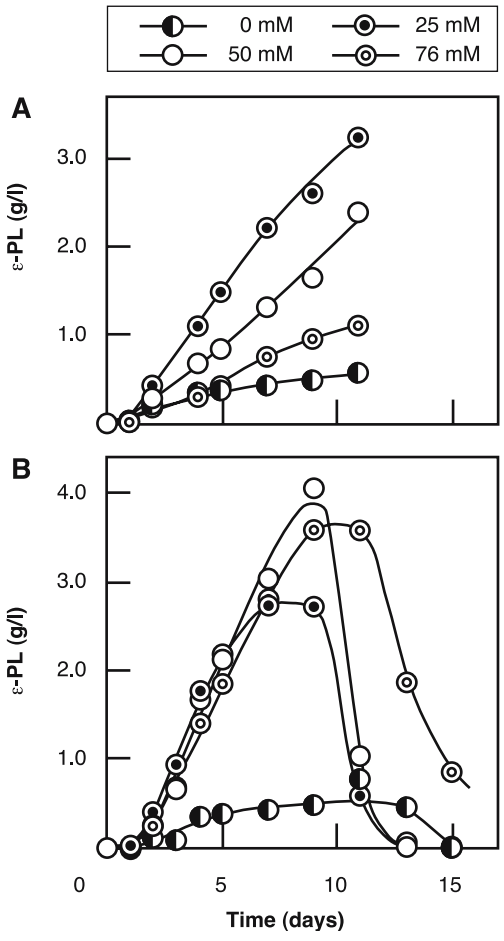

Fig. 6 Effect of concentrations of Na_2SO_4 (**a**) and $(NH_4)_2SO_4$ (**b**) on ε-PL production at pH 4.5 in USE-11. The production culture medium initially consisted of 220 mM glycerol, 95 mM citrate, 11 mM L-lysine7HCl, and 100 mM NH_4Cl besides Na_2SO_4 (**a**), and the former three components and $(NH_4)_2SO_4$ (**b**)

with USE-11 (Fig. 6). The other strain did not secrete any polymer without the addition of Na_2SO_4 or $(NH_4)_2SO_4$. This result indicates that SO_4^{2-} is decisive for the production of ε-PL in USE-51. USE-11 might to some extent store ammonium and sulfate in the cells during growth, but USE-51 may not.

Effects of external L- or D-lysine

The production rate was enhanced by the addition of L-lysine (Lys), diaminopimelate (DAP), or L-aspartate (Asp) to media containing glycerol and $(NH_4)_2SO_4$ but not citrate. The greatest enhancement was observed by Lys among the amino acids in the diaminopimelic acid pathway (DAP-P) (Fig. 7). In the case of media consisting of citrate, glycerol, and $(NH_4)_2SO_4$, the supply of adequate amounts of external Lys gave positive effects on the optimum level of ε-PL in USE-11 (Fig. 8). However, no effects were observed on the ε-PL production by the supply of 11 mM Lys in USE-51,

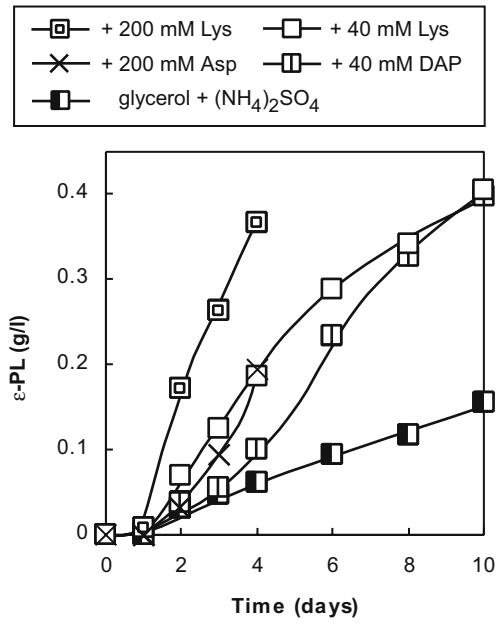

Fig. 7 Effect of amino acids in the diaminopimelic acid pathway on ε-PL production at pH 4.5 in USE-51 cells growth-cultured for 25 h. The production culture medium initially consisted of 220 mM glycerol, 76 mM $(NH_4)_2SO_4$, and 0 mM citrate besides amino acid. In the case of L-aspartate, it was difficult to maintain the medium at pH 4.5

and the ample supply of 110 mM Lys caused a great depression of less than one-tenth (Fig. 8). The D-isomer showed strong inhibitory effects in both strains (Fig. 8), and no D-isomer was incorporated into ε-PL.

Discussion

The disappearance of the polymer accompanying the pH enhancement of the culture medium was presumably caused by digestion with a peptide hydrolase(s) produced by each ε-PL producer. Such an enzyme may be a peripheral or an anchored membrane protein. Once the culture medium containing ε-PL was centrifuged from producer cells, the polymer level did not decrease even after the medium was adjusted to pH 8. The polymer diminished when the filtrate of a neutral pH range was again kept in contact with the cultured cells.

In bacteria, Lys is synthesized through DAP-P. DAP is formed via Asp produced by combining oxaloacetate (OXA) in TCA-C with the ammonium ion of a nitrogen source. The results in Fig. 7 indicate that Lys, the residue of ε-PL, is the direct monomer precursor of the biosynthesis of ε-PL (see Fig. ESM-3). The addition of citrate to the production medium facilitates the conversion of OXA to Asp through the inhibition of the cycle-forming reaction to citrate. The addition of succinate, in contrast, inhibits reactions from fumarate to L-malate (Wigler and Alberty 1960) as well as from OXA to Asp (Michuda and Martinez-Carrion 1970). The inhibition of the former reaction is fatal to TCA-C which plays a central role in the cellular metabolism. Hence, no production by the addition of succinate is understandable.

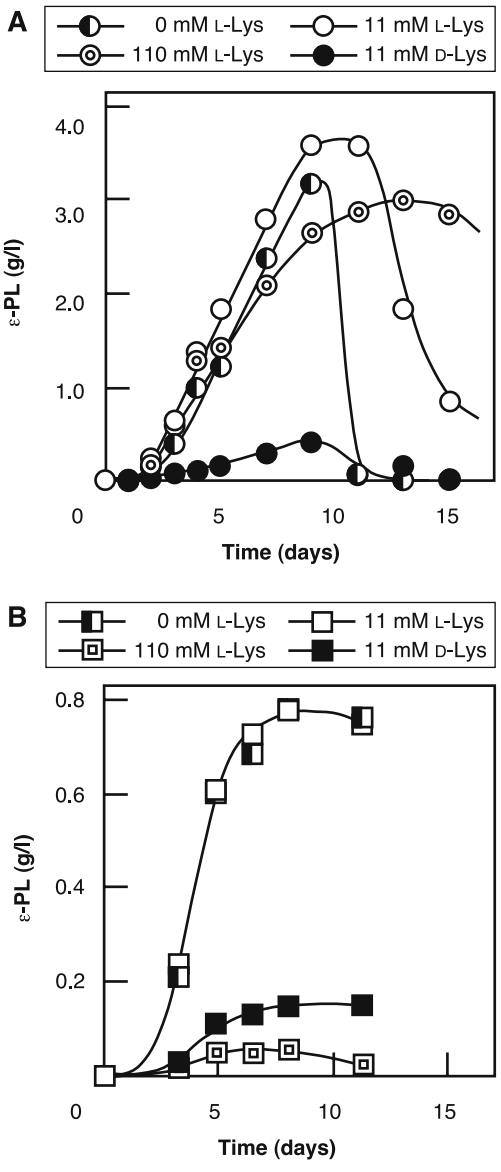

Fig. 8 Effect of initial concentration of L- or D-lysine7HCl on ε-PL production in USE-11 cells growth-cultured for 36 h (**a**) and USE-51 cells growth-cultured for 25 h (**b**). The production culture medium initially consisted of 220 mM glycerol, 95 mM citrate, and 76 mM $(NH_4)_2SO_4$ besides L- or D-lysine7HCl

The results in Fig. 8 may lead to the following discussion. USE-11 has a great metabolic flow in the Lys supply and even a greater run in ε-PL synthesis than in the former supply. The strain can utilize external Lys for the synthesis of ε-PL to a considerable extent. Thus, the supply of Lys may be limiting in the overall ε-PL production. On the other hand, USE-51 has a small flow in Lys synthesis and a low ε-PL-synthesizing activity and cannot utilize external Lys.

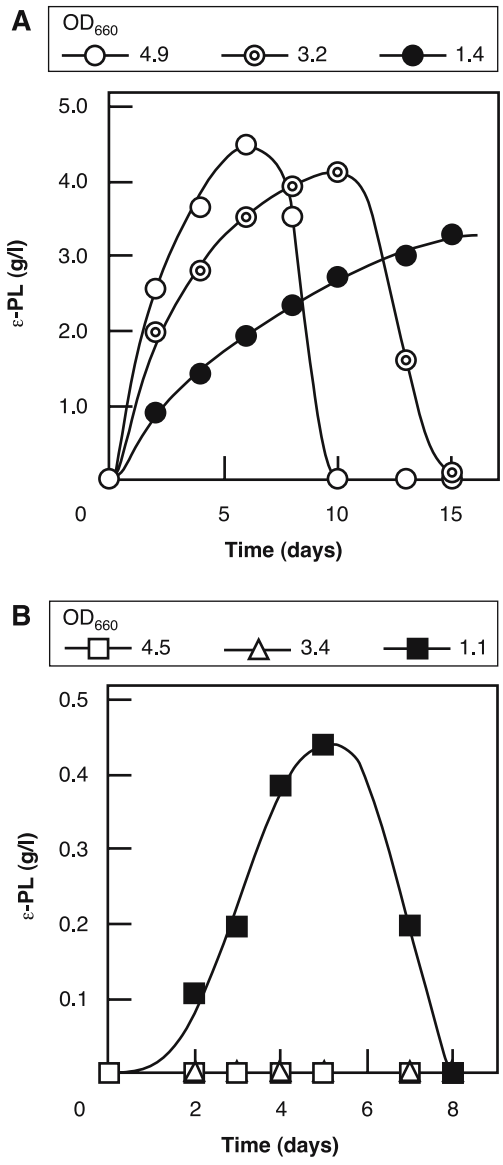

Fig. 9 Effect of cell density on ε-PL production in USE-11 (**a**) and USE-51 (**b**). Cell density was represented as the optical density (OD) of the initial production medium at 660 nm (initial turbidity). The OD was adjusted for each production medium after centrifugation of cells growth-cultured for 38 h

Polymer synthesis from the internal Lys may be limiting in the overall production in this strain. The discussion in this study is compatible with the observation that an addition of 110 mM external Lys greatly reduced polymer production in USE-51 but only slightly in USE-11. The great reduction in the former might have resulted from the inhibition of DAP-P enzymes including aspartate kinase by the external Lys.

It is not easy to obtain a plausible answer as to why the optimum level did not increase upon the successive feeding of glycerol and continuous culture at pH 4.5 (see Fig. ESM-1). The hypothesis that the production and the digestion of the polymer balanced out in the culture medium may be easily ruled out by the fact that peptide hydrolase activity was not practically detected at pH 4.5. Production cultures in media containing 4 or 0.5 g l^{-1} ε-PL with USE-11 or USE-51 gave the same production curves as those shown in Fig. 2a or b, respectively, excluding the possibility that production levels were determined through feedback inhibition by the final product of ε-PL. This shows a certain contrast with the production of some antibiotics where a final product exhibited feedback inhibition (Martin and Demain 1980). It is presumed that the optimum level of the production may be dependent upon the inherent ε-PL synthesis machinery, as was partially discussed above.

The biosynthesis of ε-PL appeared to be controlled much more strictly in USE-51 than in USE-11. From the results showing that the production depended sharply upon cell growth phase (Fig. 3b), we have examined the cell density-dependent production of the polymer. The 38 h growth-cultured cells of USE-51, which would not yield ε-PL, produced the polymer when the cell density represented as turbidity at 660 nm was reduced to one-fourth of the original turbidity of 4.5 (Fig. 9b). Results indicate that the ε-PL production by this strain was sharply dependent upon the cell density rather than the growth phase. In contrast, USE-11 produced ε-PL almost independently of the cell density and yielded polymer in a rate proportional to the density (Fig. 9a). It is known that quorum sensing system which controls gene expression in response to cell density (Demain 1998; March and Bentley 2004) regulates the production of secondary metabolites such as antibiotics and morphological differentiation into aerial mycelia and spores in *Streptomyces* (McGowan et al. 1999; Kleerebezem and Quadri 2001; Núñez et al. 2003).

The observation that SO_4^{2-} was essential to the ε-PL synthesis in the production culture might indicate that the sulfur atom would be used as a part of a cosubstrate in the polymerization reactions including the activation of Lys. The occurrence of adenylation of Lys was recently suggested in the first stage of ε-PL biosynthesis in a cell-free system of the *S. albulus* strain (Kawai et al. 2003). The adenylated lysine might then form an aminoacyl thioester with the cosubstrate of a thiol compound derived from SO_4^{2-} in the production medium. Such a mechanism would be somewhat similar to that of the biosynthesis of certain peptide antibiotics by the thiotemplate mechanism (Kleinkauf and von Döhren 1996; Finking and Marahiel 2004; Walsh 2004). However, we should stop speculating at this stage.

Finally, it should be emphasized that USE-11 is a most useful and advantageous strain from the viewpoints of basic research as well as technical applications. This strain not only shows high productivity but also the simple isolation of the product because ε-PL is a predominate component among the secreted proteins in the culture media (Fig. 2). The lack of a severe

control over the production system in this strain would also help the isolation of ε-PL-synthesizing enzymes and facilitate the cloning of encoding genes. In addition, Fig. 2 appears to indicate the occurrence of an ε-PL-hydrolyzing enzyme having great activity in USE-11, so that the strain might provide an ε-PL specific hydrolase and its encoding gene. The site of such a gene is often located close to the genes of the biopolymer-synthesizing enzymes (Rehm and Steinbüchel 2002; Ashiuchi et al. 2003; Suzuki and Tahara 2003).

Acknowledgements We thank J. Hiraki and M. Hatakeyama of Chisso (Tokyo, Japan) for providing purified ε-PL samples, and Y. Hirose of Amano Enzyme (Nagoya, Japan) for the gift of Protease A.

References

Ashiuchi M, Misono H (2002) Biochemistry and molecular genetics of poly-γ-glutamate synthesis. Appl Microbiol Biotechnol 59:9–14

Ashiuchi M, Misono H (2003) Poly-γ-glutamic acid. In: Fahnestock SR, Steinbüchel A (eds) Biopolymers, vol 7. Wiley, Weinheim, Germany, pp 123–173

Ashiuchi M, Nakamura H, Yamamoto T, Kamei T, Soda K, Park C, Sung M-H, Yagi T, Misono H (2003) Poly-γ-glutamate depolymerase of *Bacillus subtilis*: production, simple purification and substrate selectivity. J Mol Catal B 23:249–250

Ashiuchi M, Shimanouchi K, Nakamura H, Kamei T, Soda K, Park C, Sung M-H, Misono H (2004) Enzymatic synthesis of high-molecular-mass poly-γ-glutamate and regulation of its stereochemistry. Appl Environ Microbiol 70:4249–4255

Ashiuchi M, Soda K, Misono H (1999) A poly-γ-glutamate synthetic system of *Bacillus subtilis* IFO 3336: gene cloning and biochemical analysis of poly-γ-glutamate produced by *Escherichia coli* clone cells. Biochem Biophys Res Commun 263:6–12

Demain AL (1998) Induction of microbial secondary metabolism. Int Microbiol 1:259–264

Finking R, Marahiel MA (2004) Biosynthesis of nonribosomal peptides. Annu Rev Microbiol 58:453–488

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

Hezayen FF, Rehm BHA, Eberhardt R, Steinbüchel A (2000) Polymer production by two newly isolated extremely halophilic archaea: application of a novel corrosion-resistant bioreactor. Appl Microbiol Biotechnol 54:319–325

Hiraki J (2000) ε-Polylysine: its development and utilization. Fine Chem 29:18–25

Hiraki J, Hatakeyama M, Morita H, Izumi Y (1998) Improved ε-poly-L-lysine production of an *S*-(2-aminoethyl)-L-cysteine resistant mutant of *Streptomyces albulus*. Seibutsu-kogaku Kaishi 76:487–493

Hiraki J, Ichikawa T, Ninomiya S, Seki H, Uohama K, Seki H, Kimura S, Yanagimoto Y, Barnett JW Jr (2003) Use of ADME studies to confirm the safety of ε-polylysine as a preservative in food. Regul Toxicol Pharmacol 37:328–340

Hirayama C, Sakata M, Nakamura M, Ihara H, Kunitake M, Todokoro M (1999) Preparation of poly(ε-L-lysine) adsorbents and application to selective removal of lipopolysaccharides. J Chromatogr B 721:187–195

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) Bergey's manual of determinative bacteriology, 9th edn. Williams & Wilkins, Baltimore, MD

Itzhaki RF (1972) Colorimetric method for estimating polylysine and polyarginine. Anal Biochem 50:569–574

Ivánovics G, Erdös L (1937) Ein Beitrag zum Wesen der Kapselsubstanz des Milizbrandbazillus. Z Immunitatsforsch 90:5–19

Kahar P, Iwata T, Hiraki J, Park EY, Okabe M (2001) Enhancement of ε-polylysine production by *Streptomyces albulus* strain 410 using pH control. J Biosci Bioeng 91:190–194

Kahar P, Kobayashi K, Iwata T, Hiraki J, Kojima M, Okabe M (2002) Production of ε-polylysine in an airlift bioreactor (ABR). J Biosci Bioeng 93:274–280

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

Kito M, Onji Y, Yoshida T, Nagasawa T (2002a) Occurrence of ε-poly-L-lysine-degrading enzyme in ε-poly-L-lysine-tolerant *Sphingobacterium multivorum* OJ10: purification and characterization. FEMS Microbiol Lett 207:147–151

Kito M, Takimoto R, Yoshida T, Nagasawa T (2002b) Purification and characterization of an ε-poly-L-lysine-degrading enzyme from an ε-poly-L-lysine producing strain of *Streptomyces albulus*. Arch Microbiol 178:325–330

Kito M, Takimoto R, Onji Y, Yoshida T, Nagasawa T (2003) Purification and characterization of an ε-poly-L-lysine-degrading enzyme from the ε-poly-L-lysine-tolerant *Chryseobacterium* sp. OJ7. J Biosci Bioeng 96:92–94

Kleinkauf H, von Döhren H (1996) A nonribosomal system of peptide biosynthesis. Eur J Biochem 236:335–351

Kleerebezem M, Quadri LE (2001) Peptide pheromone-dependent regulation of antimicrobial peptide production in Gram-positive bacteria: a case of multicellular behavior. Peptides 22:1579–1596

Kunioka M (1997) Biosynthesis and chemical reactions of poly(amino acid)s from microorganisms. J Microbiol Biotechnol 47:469–475

Kunioka M (2004) Biodegradable water absorbent synthesized from bacterial poly(amino acid)s. Macromol Biosci 4:324–329

Kunioka M, Choi H-J (1995) Properties of biodegradable hydrogels prepared by γ-irradiation of microbial poly(ε-lysine) aqueous solutions. J Appl Polym Sci 58:801–806

Maeda-Yamamoto M, Mori Y, Osada K, Murakami H (1995) Enhancement of production of IgM and interferon-β in human cell lines by poly-lysine. Biosci Biotechnol Biochem 59:1842–1845

March JC, Bentley WE (2004) Quorum sensing and bacterial cross-talk in biotechnology. Curr Opin Biotechnol 15:495–502

Martin JF, Demain AL (1980) Control of antibiotic biosynthesis. Microbiol Rev 44:230–251

McGowan SJ, Holden MT, Bycroft BW, Salmond GP (1999) Molecular genetics of carbapenem antibiotic biosynthesis. Antoine Van Leeuwenhoek 75:135–141

Michuda CM, Martinez-Carrion M (1970) The isozymes of glutamate–aspartate transaminase. Mechanism of inhibition by dicarboxylic acids. J Biol Chem 245:262–269

Nishikawa M, Ogawa K (2002) Distribution of microbes producing antimicrobial ε-poly-L-lysine polymers in soil microflora determined by a novel method. Appl Environ Microbiol 68:3575–3581

Núñez LE, Méndez C, Braña AF, Blanco G, Salas JA (2003) The biosynthetic gene cluster for the β-lactam carbapenem thienamycin in *Streptomyces cattleya*. Chem Biol 10:301–311

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

Oppermann-Sanio FB, Steinbüchel A (2003) Cyanophycin. In: Fahnestock SR, Steinbüchel A (eds) Biopolymers, vol 7. Wiley, Weinheim, Germany, pp 83–106

Rehm BHA, Steinbüchel A (2002) PHA synthases: the key enzymes of PHA synthesis. In: Doi Y, Steinbüchel A (eds) Biopolymers, vol 3a. Wiley, Weinheim, Germany, pp 173–215

Schägger H, von Jagow G (1987) Tricine–sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166:368–379

Shih I-L, Van Y-T, Shen M-H (2004) Biomedical applications of chemically and microbiologically synthesized poly(glutamic acid) and poly(lysine). Mini-Rev Med Chem 4:179–188

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

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

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

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

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

Staneck JL, Roberts GD (1974) Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. Appl Microbiol 28:226–231

Suzuki T, Tahara Y (2003) Characterization of the *Bacillus subtilis ywtD* gene, whose product is involved in γ-polyglutamic acid degradation. J Bacteriol 185:2379–2382

Urushibata Y, Tokuyama S, Tahara Y (2002) Characterization of the *Bacillus subtilis ywsC* gene, involved in γ-polyglutamic acid production. J Bacteriol 184:337–343

Walsh CT (2004) Polyketide and nonribosomal peptide antibiotics: modularity and versatility. Science 303:1805–1810

Weber J (1990) Poly(γ-glutamic acid)s are the major constituents of nematocysts in *Hydra* (*Hydrozoa*, *Cnidaria*). J Biol Chem 265:9664–9669

Wigler PW, Alberty RA (1960) The pH dependence of the competitive inhibition of fumarase. J Am Chem Soc 82:5482–5488

Williams ST, Goodfellow M, Alderson G, Wellington EMH, Sneath PHA, Sackin MJ (1983) Numerical classification of *Streptomyces* and related genera. J Gen Microbiol 129:1743–1813

Yoshida T, Hiraki J, Nagasawa T (2003) ε-Poly-L-lysine. In: Fahnestock SR, Steinbüchel A (eds) Biopolymers, vol 7. Wiley, Weinheim, Germany, pp 107–121

Yoshida T, Nagasawa T (2003) ε-Poly-L-lysine: microbial production, biodegradation and application potential. Appl Microbiol Biotechnol 62:21–26