Construction of a Genetic System for *Streptomyces albulus* PD-1 and Improving Poly(ε-L-lysine) Production Through Expression of *Vitreoscilla* Hemoglobin

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**Introduction**

Poly(ε-L-lysine) (ε-PL) is one of the four homopoly(amino acids) discovered in nature until now. It is linked by the isopeptide bond between ε-amino and α-carboxyl groups of L-lysine residues [8]. Owing to its excellent properties, including antimicrobial activity, biodegradability, water solubility, edibility, and non-toxicity to humans and the environment, ε-PL has been used in many fields, such as food, medicine, cosmetics, and electronics [2, 26, 27]. As a novel biopolymer, ε-PL is mostly synthesized by filamentous bacteria through submerged fermentation, and various studies have reported on the achievement of efficient production of ε-PL, including pH regulation, dissolved oxygen (DO) regulation, in situ adsorption, and cheap raw materials application [1, 12, 16, 24, 25, 35]. However, because of the lack of a genetic system for most ε-PL-producing strains, very little research on enhancing ε-PL biosynthesis by genetic manipulation has been reported. In this study, an effective genetic system was established via intergeneric conjugal transfer for *Streptomyces albulus* PD-1, a famous ε-PL-producing strain. Using the established genetic system, the *Vitreoscilla* hemoglobin (VHb) gene was integrated into the chromosome of *S. albulus* PD-1 to alleviate oxygen limitation and to enhance the biosynthesis of ε-PL in submerged fermentation. Ultimately, the production of ε-PL increased from 22.7 g/l to 34.2 g/l after fed-batch culture in a 5 L bioreactor. Determination of the oxygen uptake rate, transcriptional level of ε-PL synthetase gene, and ATP level unveiled that the expression of VHb in *S. albulus* PD-1 enhanced ε-PL biosynthesis by improving respiration and ATP supply. To the best of our knowledge, this is the first report on enhancing ε-PL production by chromosomal integration of the VHb gene in an ε-PL-producing strain, and it will open a new avenue for ε-PL production.

**Keywords:** Poly(ε-L-lysine), submerged fermentation, *Vitreoscilla* hemoglobin, dissolved oxygen, *Streptomyces albulus*, genetic system
increasing agitation rate will also lead to additional energy cost. Thus, an effective approach is urgently needed to solve the oxygen limitation problem in ε-PL production. With the development of genetics and molecular biology, genetic manipulation provides new strategies that can complement the traditional methods to solve some traditional chemical engineering problems in bioprocesses. However, as mentioned above, owing to the lack of a genetic system for most ε-PL-producing strains, no research on solving the oxygen limitation by genetic manipulation has yet been reported in the ε-PL production process.

_Vitreoscilla_ hemoglobin (VHb) was the first bacterial hemoglobin discovered in nature [31]. Its main function is to bind oxygen, especially under oxygen-limited conditions, and deliver the oxygen to the terminal respiratory oxidase, thus enhancing bacterial respiration and oxidative phosphorylation [4, 21]. Recent studies have demonstrated that heterogeneous expression of VHb in S. albulus can significantly enhance the production of many valuable compounds in microorganisms [11, 17, 28, 29, 39]. Thus, whether the heterogeneous expression of VHb can significantly enhance the production of many valuable compounds in microorganisms has yet been reported in the ε-PL production process.

In the present study, to open up the possibility of genetic manipulation for_S. albulus_ PD-1, we directed our efforts toward the development of a genetic system for it. By using the established system, the VHb expression cassette was integrated into the chromosome of the ε-PL-producing strain for the first time. The fermentation results indicated that the ε-PL titer was significantly improved by the expression of VHb. Furthermore, to investigate the effects of VHb on_ S. albulus_ PD-1 in submerged fermentation, the oxygen uptake rate, transcriptional levels of ε-PL synthetase gene, and ATP level were also detected. This study would open a new avenue for enhancing ε-PL biosynthesis by genetic manipulation and become a good example for solving oxygen-limited problems in submerged fermentation processes.

Materials and Methods

Microorganisms, Plasmids, and Media

_S. albulus_ PD-1 (Accession No. M2011043), a well-known ε-PL-producing strain, was employed as the wild-type strain in this study. _Escherichia coli_ ET12567 (pUZ8002) was employed as the donor in intergeneric conjugal transfer. The site-specific integration vector, pIB139, which is a pSET152 derivative with a strong constitutive _ermE*_ promoter, was used for intergeneric conjugal transfer and VHb expression.

Luria-Bertani (LB) medium (yeast extract, 5 g/l; tryptone, 10 g/l; NaCl, 10 g/l) was used for _E. coli_ cultivation. Mannitol-soy flour (MS) agar medium (mannitol, 20 g/l; soy flour, 20 g/l; agar, 20 g/l), AS-1 (soluble starch, 5 g/l; yeast extract, 10 g/l; l-arginine, 0.5 g/l; l-alanine, 0.2 g/l; Na₂SO₄, 10 g/l; NaCl, 2.5 g/l; agar, 20 g/l; pH 7.5), ISP-2 (yeast extract, 4 g/l; malt extract, 10 g/l; glucose, 4 g/l; agar, 20 g/l), and ISP-4 (soluble starch, 10 g/l; (NH₄)₂SO₄, 2 g/l; MgSO₄·7H₂O, 2 g/l; CaCO₃, 2 g/l; NaCl, 1 g/l; K₃HPO₄, 1 g/l; ZnSO₄, 1 mg/l; FeSO₄·7H₂O, 1 mg/l; MnCl₂, 1 mg/l; agar, 20 g/l; pH 7.2) media were used for conjugal transfer. Medium 3G (M3G) (glucose, 50 g/l; (NH₄)₂SO₄, 10 g/l; yeast extract, 5 g/l; KH₂PO₄, 1.36 g/l; K₃HPO₄, 0.8 g/l; MgSO₄·7H₂O, 0.5 g/l; ZnSO₄·7H₂O, 0.04 g/l; FeSO₄·7H₂O, 0.03 g/l; pH 6.8) was used for seed culture, as well as for fed-batch fermentation of _S. albulus_ PD-1.

Conjugal Transfer Method

Conjugal transfer was conducted as described previously with some modifications [15]. _E. coli_ ET12567 (pUZ8002) harboring plasmid pIB139 was grown in 10 ml of LB medium to an OD₆₀₀ of 0.45 in the presence of kanamycin (50 µg/ml), chloramphenicol (50 µg/ml), and apramycin (50 µg/ml). Cells were then washed twice to remove the antibiotics and were subsequently resuspended in 500 µl of fresh LB medium. While washing the _E. coli_ cells, _S. albulus_ PD-1 spores (about 10⁷) were suspended in 500 µl of LB medium. Subsequently, _E. coli_ donor cells and _S. albulus_ PD-1 spores were mixed thoroughly and spread on 20 ml of agar medium (MS, ISP-2, ISP-4, or AS-1) containing 10 mM MgCl₂. The plates were incubated at 30°C for about 18 h and then overlaid with 1 ml of sterile water containing 0.05 mg of nalidixic acid and 0.1 mg of apramycin. The plates were further incubated at 30°C for about 3 days, and the ex-conjugants were counted.

Construction of Recombinant Plasmid pIB139-rgb

The VHb gene (rgb) (Accession No. AF292694, gifted by the Institute of Biochemistry and Cell Biology, SIBS, CAS, China) was amplified by PCR using the following primers: P1 (5’-GGAGATTCGATATGCTGCTGAGCACGCAAC-3’, gel purified) and P2 (5’-GCTCTAGAGTATATAAACGGCTTGAGC-3’, XbaI site underlined). Subsequently, the PCR product was digested with _Ndel_ and _XbaI_ gel purified, and ligated into the corresponding sites of pIB139 to generate pIB139-rgb (Fig. 1A). The plasmid pIB139-rgb was identified by restriction digestion and DNA sequencing. Then, the identified recombinant plasmid was introduced into _S. albulus_ PD-1 via the conjugal transfer method mentioned above.

Cultivation Conditions

Submerged fermentation experiments were performed in flasks and a bioreactor. For seed cultures, a loop of 1-week-old fully grown spores was inoculated into 100 ml of M3G medium contained in a 500 ml flask and then incubated at 30°C and 200 rpm for 24 h as stock culture. To explore the VHb effects on ε-PL biosynthesis in flask fermentation, especially under oxygen-limited conditions, 10% seeds were inoculated into 45, 90, 135, and 180 ml of M3G medium in 500 ml flasks, and the yield of ε-PL was
determined after the cultivation was incubated at 30°C and 200 rpm for 72 h. Fed-batch fermentations were conducted in a 5 L bioreactor (KoBio Tech Co., Ltd., Korea) by using a two-stage pH control strategy [12, 34]. In the first stage, the pH was controlled at 6.0 for cell growth, and in the second stage, the pH was controlled at 4.0 for ε-PL biosynthesis. When the glucose concentration in the culture broth decreased to about 10 g/l, the feeding solution (glucose, 500 g/l; (NH₄)₂SO₄, 50 g/l) was pumped into the broth to keep the glucose concentration at approximately 10 g/l.

Determination of Cell Growth, Glucose Concentration, pH, DO, and ATP Level

Cell growth was measured in terms of dry cell weight (DCW). The harvested culture sample was filtered; the mycelia were washed and dried at 65°C until constant weight was achieved. Glucose concentration in the culture broth was determined by using a biosensor (SBA-40C; Shandong Science Academy, China). The yield of ε-PL was determined by high-performance liquid chromatography, following the method reported previously [34]. Intracellular ATP concentration was quantified by a chemiluminescence response method as reported before [35]. The pH and DO were measured by probes of the bioreactor. The oxygen uptake rate (OUR) of bacteria was analyzed with a process mass spectrometer (SHP8400/PMS-162R). All assays were performed in triplicate, and experimental errors were <5%.

Analysis for the Biological Activity of VHb

The CO-difference spectral analysis was performed to determine the biological activity of VHb according to the previously described method [39].

Analysis of the Transcriptional Level of ε-PL Synthetase Gene

As the fermentation process reached 110 h, the maximum specific ε-PL production rate was reached. At this moment, the transcriptional level of the ε-PL synthetase gene (pls) was determined by quantitative real-time PCR (qRT-PCR) according to the method mentioned before [35]. The relative gene transcriptional level was calculated by the 2^ΔΔCt method, with hrdB (a housekeeping sigma factor gene) as the endogenous control gene [32, 38]. For pls and hrdB, the following primers were used: pls-F, 5'-CGGATTCGTCCCAACCTCCT-3' and pls-R, 5'-GACGATGATCAGCCACCA-3'; hrdB-F, 5'-CGACTACACCAAGGGCTACA-3' and hrdB-R, 5'-TTGTTGATGACCTCGACC-3'.

Results and Discussion

Construction of a Genetic System for S. albulus PD-1

With the development of molecular biology, intensive genetic engineering applications have been applied in biotechnology [19, 20]. In such applications, the target DNA fragments should be transferred into candidate cells first. Streptomycetes are well known for their ability to produce many valuable secondary metabolites. In order to obtain the genetically modified streptomycetes, one of the key challenges is delivering the target DNA fragments into living streptomycetes cells efficiently. However, owing to the slow growth and thick cell wall of most streptomycetes, the genetic system of these organisms has obstacles compared with manipulations in E. coli, yeast, and other commonly used industrial strains [18]. With the continuous efforts of researchers, some kinds of genetic systems were developed for streptomycetes, including polyethylene glycol-mediated method, electrotransformation, and intergeneric conjugal transfer [5, 10, 18, 22, 23]. Among the three methods, intergeneric conjugal transfer has been performed in many streptomycetes because of its high transformation efficiency, and reproducibility. Thus, we attempted to construct an intergeneric conjugal transfer system for S. albulus PD-1.

It has been reported that the type of medium used has a significant effect on the efficiency of conjugation. Thus, four representative media (AS-1, ISP-2, ISP-4, and MS) were used for conjugal transfer for S. albulus PD-1 [3, 22]. As shown in Table 1, the highest transformation frequency (4.0 ± 0.5 x 10⁷ per recipient) was observed when MS medium was used for conjugal transfer. Thus, MS medium was selected as the most appropriate medium and was
employed in the subsequent experiments. To confirm whether pIB139 was integrated into the chromosome of *S. albulus* PD-1, the apramycin resistance gene was amplified from the chromosome of transformants, but not from wild-type strain *S. albulus* PD-1 (data not shown). In particular, the integration of plasmid pIB139 had no effect on cell growth and ε-PL production of *S. albulus* PD-1, and it was stable after passing several generations. In previous studies, Hamano *et al.* [9] made a lot of effort to construct the genetic system for *S. albulus* IFO 14147, which is the only ε-PL-producing strain owning the genetic systems. In their studies, they spent much time constructing free-replicating plasmids for *S. albulus* IFO 14147. Even though the free-replicating plasmids have many uses for genetic operation, stable maintenance of a free-replicating plasmid requires selection via expensive antibiotics. Compared with free-replicating plasmids, the plasmid pIB139 (capable of integration into ϕ31 attB site in *S. albulus* PD-1) could be more stable in streptomycetes, which would not only reduce the cost of additional antibiotics but also prevent the contamination caused by antibiotics. Thus, pIB139 was used for VHb expression in the following work.

### Construction of *S. albulus* PD-2

For heterologous expression of VHb in *S. albulus* PD-1, the *vgb* was cloned and placed under the control of ermE* promoter in plasmid pIB139 to create pIB139-vgb (Fig. 1A). Subsequently, the plasmid pIB139-vgb was introduced into *S. albulus* PD-1 via the established genetic system. The ex-conjugant was designated as *S. albulus* PD-2, which was verified by PCR amplification by using the primer pair P1/P2.

A PCR product of 450 bp was obtained during electrophoresis when chromosomal DNA from *S. albulus* PD-2 was used as the PCR template, whereas no band was observed when chromosomal DNA from *S. albulus* PD-1 was used (Fig. 1B). This observation confirmed that the *vgb* had been successfully integrated into the chromosome of *S. albulus* PD-2.

The biological activity of VHb was measured using the CO-difference spectra. The CO-difference spectra showed a significant VHb CO-binding absorbance peak at 420 nm from the crude cell extract of *S. albulus* PD-2 compared with that of the wild-type strain (Fig. 2). This typical peak of VHb protein demonstrated that the expressed VHb in *S. albulus* PD-2 was biologically active [17, 39]. Taken together, by using the established genetic system, the biologically active VHb was successfully expressed in *S. albulus* PD-2.

### Comparison of ε-PL Production for Different Loading Volumes in Flask Culture

Heterologous expression of VHb has been demonstrated in many strains to improve cell growth and secondary metabolite productivity, especially under oxygen-limited conditions [33, 39]. To reveal the effect of VHb expression on ε-PL production, *S. albulus* PD-1 and *S. albulus* PD-2

<table>
<thead>
<tr>
<th>Liquid volume (ml in 500 ml flask)</th>
<th>DCW (g/l)</th>
<th>Yield of ε-PL (g/l)</th>
<th>ε-PL increase rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. albulus</em> PD-1</td>
<td><em>S. albulus</em> PD-2</td>
<td><em>S. albulus</em> PD-1</td>
</tr>
<tr>
<td>50</td>
<td>10.36 ± 0.31</td>
<td>11.27 ± 0.26</td>
<td>1.21 ± 0.07</td>
</tr>
<tr>
<td>100</td>
<td>9.92 ± 0.24</td>
<td>10.58 ± 0.27</td>
<td>0.91 ± 0.05</td>
</tr>
<tr>
<td>150</td>
<td>8.95 ± 0.17</td>
<td>9.91 ± 0.21</td>
<td>0.76 ± 0.04</td>
</tr>
<tr>
<td>200</td>
<td>8.52 ± 0.21</td>
<td>9.43 ± 0.17</td>
<td>0.43 ± 0.02</td>
</tr>
</tbody>
</table>

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**Table 1.** Effect of medium type on the intergeneric conjugation efficiency.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Transformation frequency</th>
</tr>
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<tbody>
<tr>
<td>AS-1</td>
<td>((6.0 \pm 0.3) \times 10^7)</td>
</tr>
<tr>
<td>ISP-2</td>
<td>((2.0 \pm 0.5) \times 10^8)</td>
</tr>
<tr>
<td>ISP-4</td>
<td>0</td>
</tr>
<tr>
<td>MS</td>
<td>((4.0 \pm 0.5) \times 10^7)</td>
</tr>
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**Table 2.** Summary of ε-PL yield in different volumes of culture broth.

**Fig. 2.** CO-difference spectral analysis of crude extracts of *S. albulus* PD-1 and *S. albulus* PD-2.
were cultured in 500 ml flasks first, with increasing loading volumes from 50 ml to 200 ml. As shown in Table 2, the loading volume had a strong effect on cell growth and ε-PL production. In all these cases, the concentration of ε-PL and DCW decreased with the increasing loading volume. According to a report, the increasing liquid volume decreases the volumetric oxygen mass transfer rate and leads to oxygen limitation [30]. This phenomenon could be the main reason for the decrease in ε-PL production and cell growth. However, the expression of VHb in S. albulus PD-2 was functional for reducing the unsatisfactory effect caused by the high liquid volume. In addition, when the liquid volume was increased, the effect of VHb was more significant. With 50 ml of broth in a 500 ml flask, the expression of VHb could only contribute to 0.16-fold increase of ε-PL titer. However, with 200 ml of broth in a 500 ml flask, the expression of VHb could lead to as much as 1.28-fold increase of ε-PL titer. Thus, our results supported that VHb expression in S. albulus PD-2 can solve the problems caused by limited oxygen and eventually increase ε-PL production.

Performance of Fed-Batch Operation Using Recombinant Strain

To verify the feasibility of VHb expression in actual production, S. albulus PD-1 and S. albulus PD-2 were further incubated in a 5 L bioreactor. As illustrated in Fig. 3C, for both strains, the DO concentrations in the culture broth decreased continuously at the early stage of the fermentation period. Finally, the DO concentration maintained at about 23.5% after 55 h, compared with 18.9% after 52 h in S. albulus PD-2 fermentation process. In another words, the DO level in the culture broth of S. albulus PD-2 was lower compared with that in S. albulus PD-1. A similar phenomenon has been reported for S. diastatochromogenes [17]. In previous studies, researchers found that VHb can increase the intracellular effective DO concentrations by allowing a more effective intracellular delivery of oxygen [6, 28]. The lower DO in S. albulus PD-2 culture broth may be because more oxygen was transported to cells and metabolized rapidly. Therefore, the oxygen uptake rate (OUR) values of the wild-type strain and of S. albulus PD-2 were determined. As depicted in Fig. 3D, the OUR values of S. albulus PD-2 were higher than those of the wild-type strain regardless of the limited DO concentration during the 55th–168th h or not (0–55 h). The high OUR supported the increased respiration of the recombinant strain with VHb expression. The enhancement of respiration will provide more energy for both cell growth and ε-PL biosynthesis. These results explained the growth advantage (Fig. 3B) and high ε-PL production (Fig. 3A) exhibited by S. albulus PD-2.

Ultimately, S. albulus PD-2 obtained a final biomass concentration of 33.4 g/l and ε-PL of 34.2 g/l, which corresponded to 27.5% and 50.7% increase, respectively, compared with the wild strain. Moreover, the specific ε-PL production rate of S. albulus PD-2 was higher than that of the wild-type strain (Fig. 3E), indicating that the improvement of ε-PL production was not only due to the higher biomass volume of S. albulus PD-2 (Figs. 3B and 3F) but that the expression of VHb also strengthened the ε-PL synthesis ability of the signal cell. Recently, oxygen-vectors were added in culture broth to alleviate the oxygen-limited problem in the fermentation process of S. albulus PD-1 [35]. As a result, 30.8 g/l of ε-PL could be produced with 0.5% n-dodecane in the culture broth. Compared with oxygen-vector applications, the expression of VHb not only resulted in a higher ε-PL production but also saved the cost brought by oxygen-vectors addition. Thus, the expression of VHb provides a new strategy that can complement the existing methods for alleviating low DO concentrations in the ε-PL production process.

Changes of ATP Level in S. albulus PD-2

Until now, several hypotheses have been raised to elucidate the function of VHb. The dominant view at present is that VHb could bind oxygen and deliver the oxygen to the terminal respiratory oxidase and/or oxygenases, thus enhancing bacterial respiration and oxidative phosphorylation [4, 14, 21]. In short, VHb can take part in one or more steps of the respiratory chain and thus promote respiration of bacteria. The increase in OUR of S. albulus PD-2 agreed with those perspectives, and the enhancement of respiratory action may probably lead to a higher ATP, which is an essential cofactor in ε-PL biosynthesis. To verify this speculation, the ATP level was also determined during the fermentation process.

Fig. 4 shows the ATP level of S. albulus PD-2 and the wild-type strain. Amongst the variation trends, the ATP level in S. albulus PD-2 was higher than that of the wild-type strain at all times. The fact that high ATP level is a benefit for ε-PL synthetase (Pls) has been demonstrated in some ε-PL-producing strains [35, 36]. As reported, ATP regulates ε-PL in two ways: first, ATP is directly involved in the assembly of ε-PL through activating lysine to llysyl-O-AMP [37]; second, a high ATP level is essential for the expression of pls [36]. The increase of transcriptional level of pls in S. albulus PD-2 also validated the improvement of ATP level indirectly (Fig. 5). Thus, we hold the opinion that
with the expression of VHb in *S. albulus* PD-2, the respiratory action was enhanced and more ATP was generated. The high ATP level in *S. albulus* PD-2 stimulated ε-PL biosynthesis by improving the transcriptional level of *pls* and activating more lysine to participate in ε-PL assembly.

In conclusion, in the present study, we constructed a genetic system for *S. albulus* PD-1. By using the established genetic system, *vgb* was integrated into the chromosome of *S. albulus* PD-1 for the first time. The expression of VHb in *S. albulus* PD-1 relieved the unsatisfactory effect caused by limited oxygen in the culture broth, and significantly enhanced ε-PL production compared with the wild-type strain. By measuring the oxygen uptake rate, the transcriptional

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**Fig. 3.** Time curve analysis of ε-PL production (A), cell growth (B), DO concentration (C), OUR (D), specific ε-PL production rate (E), and specific cell growth rate (F) of *S. albulus* PD-1 and *S. albulus* PD-2 in a 5 L bioreactor culture experiment.
level of $\text{pls}$, and ATP level, it can be concluded that the expression of VHB in \textit{S. albulus} PD-1 enhanced $\varepsilon$-PL biosynthesis through improving respiration and the ATP supply. We believe that this study will open a new avenue for $\varepsilon$-PL production by genetic engineering.

**Acknowledgments**

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