Hyper-production of porcine contagious pleuropneumonia subunit vaccine proteins in Escherichia coli by developing a bicistronic T7 expression system

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Abstract

The ApxII toxin and outer membrane lipoprotein (Oml) of Actinobacillus pleuropneumoniae (A. pleuropneumoniae) are vital vaccine antigens against porcine contagious pleuropneumonia (PCP), a prevalent infectious disease in the swine industry worldwide. Previous studies have reported the recombinant expression of ApxII and Oml in Escherichia coli (E. coli). However, their yields were not satisfactory. Here, we aimed to enhance the production of ApxII and Oml in E. coli by constructing a bicistronic expression system based on the widely used T7 promoter. To create efficient T7 bicistronic expression cassettes, 16 different fore-cistron sequences were introduced downstream of the T7 promoter. The four most potent expression vectors were screened, and the expression of three vaccine antigens Oml1, Oml7, and ApxII in these four bicistronic vectors were enhanced compared to the monocistronic control. Further optimization of the fermentation conditions in micro-well plates led to improved production of Oml1, Oml7, and ApxII. Finally, the production yields reached unprecedented levels of 2.43 g/L, 2.59 g/L, and 1.21 g/L, respectively, in a 5 L bioreactor. These three antigens also demonstrated well-protective immunity against A. pleuropneumoniae infection. In conclusion, this study established a highly efficient bicistronic T7 expression system and achieved the hyper-production of PCP vaccine proteins. This bicistronic T7 expression system could be a valuable tool for the improved production of other proteins, especially recombinant vaccines, in E. coli.

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Abstract

The ApxII toxin and outer membrane lipoprotein (Oml) of *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) are vital vaccine antigens against porcine contagious pleuropneumonia (PCP), a prevalent infectious disease in the swine industry worldwide. Previous studies have reported the recombinant expression of ApxII and Oml in *Escherichia coli* (*E. coli*). However, their yields were not satisfactory. Here, we aimed to enhance the production of ApxII and Oml in *E. coli* by constructing a bicistronic expression system based on the widely used T7 promoter. To create efficient T7 bicistronic expression cassettes, 16 different fore-cistron sequences were introduced downstream of the T7 promoter. The four most potent expression vectors were screened, and the expression of three vaccine antigens Oml1, Oml7, and ApxII in these four bicistronic vectors were enhanced compared to the monocistronic control. Further optimization of the fermentation conditions in micro-well plates led to improved production of Oml1, Oml7, and ApxII. Finally, the production yields reached unprecedented levels of 2.43 g/L, 2.59 g/L, and 1.21 g/L, respectively, in a 5 L bioreactor. These three antigens also demonstrated well-protective immunity against *A. pleuropneumoniae* infection. In conclusion, this study established a highly efficient bicistronic T7 expression system and achieved the hyper-production of PCP vaccine proteins. This bicistronic T7 expression system could be a valuable tool for the improved production of other proteins, especially recombinant vaccines, in *E. coli*.

Keywords: bicistronic T7 expression system, PCP subunit vaccine protein, hyper-production, *Escherichia coli*, recombinant expression

Introduction

*Actinobacillus pleuropneumonia* (*A. pleuropneumonia*) is the causative agent of porcine contagious pleuropneumonia (PCP), a highly contagious and usually fatal disease of pigs.\cite{1,2} It has caused considerable economic losses to the global pig-rearing industry and is also one of the five internationally recognized diseases that endanger the pig industry. Although antibiotics can prevent PCP outbreaks, the abuse of antibiotics has led to drug resistance of pathogenic bacteria, and the policy of restrictive antibiotics in livestock and poultry feeding products in many countries drives up the demand for PCP vaccines.\cite{3,4} Currently, the PCP vaccines mainly used are whole cell inactivated vaccines of several pathogenic serotypes, with poor-cross protection.\cite{5} Recently, subunit vaccine derived from the immune-protective antigens of *A. pleuropneumoniae*, such as Apx toxins and outer membrane lipoprotein (Oml), common to many serotypes of *A. pleuropneumonia* strains, have demonstrated strong cross-immune effect and good prospects for preventing PCP.\cite{5,6} Among the numerous Apx toxins, ApxII is the most promising vaccine candidate, as all 15 serotypes of *A. pleuropneumoniae* except serotype 10 express it.\cite{6,7} So far, laboratories and industry have successfully cloned and expressed Oml and ApxII in *E. coli*, yeast, *Corynebacterium glutamicum* (*C. glutamicum*), etc.\cite{8–12} However, *E. coli* is still the most widely used expression system for the production of PCP subunit vaccine proteins, and the current recombinant expression in *E. coli* exists some drawbacks that need to be further improved. For example, protein yields are low, especially for ApxII.\cite{10} Most recombinant ApxII existed in an inactive inclusion body form, resulting in complicated downstream work and substantial loss of the target protein. Therefore, the efficient expression of PCP subunit vaccine proteins in *E. coli* remains a huge challenge.

To improve the production of recombinant proteins in *E. coli*, several strategies have been adopted by researchers, including developing strong promoters and ribosome binding sites (RBSs), modifying host cells, and optimizing fermentation conditions, etc.\cite{13–16} Among them, the operation of promoters is considered the most effective way, as promoters confer direct control of the mRNA abundance for protein translation and can determine nearly 80% of the protein expression level. To date, promoters from different origins, such as bacteria (Lac, tac, trp, and araBAD) and bacteriophage (T7, T5, and SP6), have been developed and applied in *E. coli*.\cite{14} Due to its outstanding performance, pET expression vector based on the T7 promoter is still the most widely used and popular commercial expression system.\cite{14,17} According to statistics, more
than 90% of the 2003 PDB proteins were produced using the T7 promoter.\cite{17}

Despite the effectiveness of strong promoters in enhancing gene expression, other genetic elements, such as unfavorable mRNA secondary structures in the translation initiation region (TIR), can also limit the expression level of target genes.\cite{18} Optimizing the TIR region for each target gene can solve this problem but is cumbersome and time-consuming.\cite{19} Alternatively, the bicistronic design (BCD) can be utilized to overcome this issue.\cite{20} In the BCD expression cassette, a short coding sequence (CDS) is inserted as the fore-cistron upstream of the target gene, the intrinsic helicase activity of ribosome recruited by the translation of fore-cistron can disrupt the mRNA structure around the target gene TIR, thus facilitating its translation initiation.\cite{20,21}

Since the fore-cistronic peptide has almost no functional purpose in BCD, the fore-cistron can be flexibly modified and replaced as an independent genetic element. A well-translated fore-cistron can be introduced as an “enhancer” to further improve the expression level of the target protein through translation coupling. For example, in our previous study, the expression of seven protein models was greatly enhanced in C. glutamicum by using bicistronic P\textsubscript{lac} systems containing well-performed fore-cistron sequences.\cite{19} Although BCD has proven effective in many prokaryotic expression systems, given the complexity of the expression frame, constructing a new bicistronic system to enhance recombinant protein expression is not a straightforward task. Sequences that allow efficient ribosome translation are not always suitable as fore-cistrons.\cite{19,22,23} Therefore, to ensure that the newly constructed BCD system effectively enhances protein production, optimizing the genetic elements, especially the fore-cistron sequence, is necessary.

In this study, to achieve the hyper-production of PCP subunit vaccine proteins in E. coli, we built the bicistronic T7 expression system. We evaluated the effect of a series of fore-cistron sequences on BCD expression intensity and selected the top four strongest BCD vectors for the expression of three PCP vaccine proteins, Oml1, Oml7, and ApxII. Among them, Oml1 and Oml7 are the outer membrane lipoprotein of A. pleuropneumoniae serotypes 1 and 7 frequently appeared in China. Optimal culture conditions, induction conditions, and medium composition were also established to further improve protein yields. Finally, fed-batch cultivation in a 5 L bioreactor resulted in unprecedented high yields of Oml1 (2.43 g/L), Oml7 (2.59 g/L), and ApxII (1.21 g/L), and these recombinant antigens exhibited good immune protective efficacy in mouse models.

2. Materials and methods

2.1 Strains and culture condition

The strains and plasmids used in this study are shown in Table S1. E. coli JM109 is used for plasmid construction, and BL21 is used for protein expression. E. coli JM109 was cultured in LB medium or on LB plate containing 1.5% (w/v) agar at 37 °C. E. coli BL21 was cultured in LB medium or TBSB medium (yeast extract 24 g, tryptone 12 g, KH\textsubscript{2}PO\textsubscript{4} 2.31 g, K\textsubscript{2}HPO\textsubscript{4}·3H\textsubscript{2}O 12.55 g, glycerin 10 g, and 1 L H\textsubscript{2}O) at 37 °C. The final concentration of kanamycin for E. coli JM109 and BL21 is 50 mg/L.

2.2 DNA manipulation and Plasmid construction

Plasmid extraction and PCR purification used kits from CWBIO. The polymerase chain reaction (PCR) was carried out using EsTaq polymerase (CWBIO, China) or PrimerSTAR (TaKaRa, China). The restriction endonuclease was purchased from TaKaRa. Gene sequences were inserted into target vectors through ligation reaction (TaKaRa, China) or homologous recombination (Abclone, China). All experiments were performed following the standard procedure.

Table S2 lists all the primers used in this study. First, the EGFP sequence was inserted into pET28a through Nco I and Hin d III to obtain pET28-EGFP. This plasmid was used as a skeleton to construct bicistronic T7 expression vectors containing different fore-cistronic sequences (Fig. 1A). The construction process was as follows: The 62 bp fore-cistron sequence ended with a conserved SD2 (AAAGGAGGACAACTAATG) and TAATG translation coupling frame were obtained by primer annealing. Then, pET28-EGFP was digested withNco I, and these sixteen different sequences were inserted upstream of EGFP through homologous recombination, respectively. TheNco I cleavage site was removed from the final plasmid (Fig. 1B).
To construct expression plasmids for PCP vaccine protein production, the Oml1, Oml7, and ApxII genes were codon-optimized and synthesized by Genewiz (Suzhou, China). The sequences were listed in Supplementary material, and all three genes were appended with a 6xhis tag at the 3-terminal. Then, these gene sequences and the corresponding fore-cistron sequences were inserted into the pET28a to obtain BCD expression vectors. The expression plasmid containing a monocistronic T7 promoter was used as a control.

2.3 EGFP intensity measurement

After successful construction in E. coli JM109, all plasmids were transformed into E. coli BL21. Three transformants were picked up for each expression strain and activated in 24 deep-well plate containing 2 mL LB medium and 50 mg/L kanamycin at 37°C, 220 rpm for 12 h. Then, the cultures were transferred to 2 mL fresh LB medium with a 10% (v/v) inoculation rate, and 1 mM IPTG was directly added after inoculation. After 24 h of induction, the cells were collected and diluted at about 0.5. The fluorescence intensity was measured by a fluorescence spectrophotometer at a 488 nm excitation wavelength and a 507 nm emission wavelength. Each sample’s unit fluorescence intensity was calculated by normalizing the fluorescence intensity by OD_{600}.

2.4 Real-time quantitative PCR (qPCR) and calculation of relative translation efficiency

To compare the EGFP transcriptional level of different plasmids, E. coli strains were in LB medium as described above. 1 mL of cultures was collected by centrifugation and washed twice with ice-cooled PBS. Total RNA extraction (CWBIO, China), reverse transcription (Vazyme, China), and qPCR (Vazyme, China) were then performed according to the manufacturer’s instructions. qPCR was carried out using StepOnePlus (Applied Biosystem, USA) system and the condition was set as follows: 95 degC for 30 s and 40 cycles at 95 degC for 15 s, 62 degC for 30 s, and 72 degC for 20 s. Each sample contains three biological repeats and three duplicated wells, the relative EGFP transcription level was analyzed by the 2^{-\Delta\Delta C_t}\text{method}[^{19}]. The 16S rRNA was used as the endogenous control and the EGFP transcription level of pET28-EGFP was defined as 1. The relative EGFP translation efficiency is calculated by dividing the EGFP fluorescence intensity by mRNA abundance as previously reported[^{24,25}].

2.5 Optimization of PCP vaccine protein production in micro-well plates (MWP)

Optimization of expression vector

PCP vaccine protein genes were cloned into different expression vectors. To compare the expression performance of these vectors, three transformants were picked up for each strain and activated in MWP containing 2 mL LB medium and 50 mg/L kanamycin at 37°C, 220 rpm for 12 h. Then, the cultures were transferred to 2 mL fresh TBSB medium with a 10% (v/v) inoculation rate, and 1 mM IPTG was directly added after inoculation. After 24 h of induction, the cells were collected and disrupted for SDS-PAGE analysis.

Optimization of cultivation temperature

The cultivation conditions were set according to the above description. After the addition of IPTG, MWP was kept at 25, 30, 37, and 220 rpm for 24 h, respectively.

Optimization of induction conditions

For the optimization of IPTG concentration, after the culture was transferred to TBSB, IPTG with a final concentration of 0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mM was added to the culture medium. Then, MWP was kept at 25 and 220 rpm for 24 h.

For the optimization of the pre-induction period, three transformants were picked up for each strain and activated in MWP containing 2 mL LB medium and 50 mg/L kanamycin at 37°C, 220 rpm for 12 h. Then, the cultures were transferred to 2 mL fresh TBSB medium with a 10% (v/v) inoculation rate. 0.1 mM IPTG was added to the culture medium at 0, 1, 2, 3, and 4 h after inoculation, respectively. All MWPs were kept at 25, 220 rpm for 24 h.

Optimization of medium components
TBSB was used as the initial medium for medium components optimization. For carbon source optimization, 10 g/L glucose, fructose, xylose, and sucrose were added into the TBSB medium to replace the original glycerin. The cell growth and protein production using glucose carbon source were used as control. For nitrogen source optimization, additional 10 g/L urea, \((\text{NH}_4\text{)}_2\text{SO}_4\), and \(\text{NH}_4\text{Cl}\) was added to the TBSB medium, and the protein yield and OD_{600} in the TBSB medium were used as control. For all medium optimization experiments, the cultivation and induction condition was set at the optimal level previously determined.

2.6 SDS-PAGE analysis and protein quantitation

After 24 h cultivation, 10 OD_{600} of cells harboring PCP vaccine proteins were harvested by centrifugation at 12,000g, 4 °C for 10 min. Cells were washed and resuspended with 1 mL PBS, and then disrupted by sonication on ice. After sonication, the lysates were centrifuged at 12,000g for 15 min and the supernatants were collected for 12% (w/v) SDS-PAGE analysis. The concentration of the target protein band was determined by measuring and comparing its strength with that of standard bovine serum albumin (BSA) at a known concentration loaded in the same gel (Image J software).

2.7 Fed-batch cultivation and purification of PCP antigen proteins

The fed-batch culture was carried out in a 5 L bioreactor (Applikon, Holland) with a working volume of 2 L. After overnight activation in LB medium, 200 mL \(\text{E. coli}\) seed solution was transferred to 1.8 L TBSB medium. Throughout the fermentation process, the dissolved oxygen was maintained at 30% (v/v). pH was controlled at 7.0, and the stirring speed was set at 400-1000 rpm. The temperature was first maintained at 37 °C for 2 h and then changed to 25 °C after IPTG addition. The induction conditions refer to the optimum level determined in MWP. To avoid starvation, a feed medium (50 g/L yeast extract, 714 g/L glycerin, and 25 g/L \((\text{NH}_4\text{)}_2\text{SO}_4\)) was added with a 3.5 mL/h/L feeding rate after 12 h of inoculation. The antigen protein was purified using an AKTA purifier system (GE, Sweden) and a HisTrap HP affinity column. The purity of the purified protein was then determined by SDS-PAGE analysis.

2.8 Challenge experiment with \(\text{A. pleuropneumoniae}\) serotype 7

The \(\text{A. pleuropneumoniae}\) serotype 7 strain used in this study was provided by Tecon Biology Co., Ltd and was cultured in BHI medium supplemented with 10 mg/L \(\beta\)-nicotinamide adenine dinucleotide at 37 °C.[26] For the challenge experiment, forty mice were divided into four groups: Three experimental groups were immunized with 20 \(\mu\)g purified Oml1, Oml7, or ApxII, respectively. The control group was immunized with PBS. After 14 days of immunization, all mice were intraperitoneally injected with 0.5 mL of \(\text{A. pleuropneumoniae}\) serotype 7 (6\( \times \)10^8 CFU). The survival of mice was counted in the following 7 days.

3. Results

3.1 Construction of the bicistronic T7 expression system

The artificial bicistronic structure in prokaryotes has been shown to enhance the production of recombinant proteins.[20] In this study, to further improve the expression performance of the T7 promoter in \(\text{E. coli}\), we constructed the T7 in a bicistronic manner. We selected 8 well-performed fore-cistrons from the \(\text{P}_{\text{btac}}\) system based on our previous study in \(\text{C. glutamicum}\),[19] and optimized their sequences according to the codon preference of \(\text{E. coli}\), thus generating 8 additional sequences. In total, 16 fore-cistron sequences were used to construct bicistronic T7 systems, and the sequence information was presented in Table 1. According to the characteristics of the BCD expression cassette, a conserved SD sequence (the second SD motif, SD2) and a translation coupling frame were inserted between the fore-cistron and the reporter gene EGFP (Fig. 1A and 1B).[20] The LacI gene located upstream of the T7 promoter confers the inducible characteristic of the BCD system, so the expression of recombinant proteins in bicistronic T7 systems requires the addition of IPTG.

3.2 Characterization of the effect of different fore-cistron sequences on the performance of bicistronic T7 expression systems
The expression plasmid was constructed in *E. coli* JM109 and later transferred into *E. coli* BL21, a more efficient strain for recombinant protein expression. To evaluate the performance of different fore-cistron sequences in the bicistronic T7 expression system, EGFP fluorescence intensity was measured and standardized with OD\textsubscript{600} after 24 h of cultivation in MWP. The EGFP expression level in pET28-EGFP with a monocistronic T7 served as the control. The results in Fig. 2A showed that extensive fluorescence intensity of EGFP was observed in bicistronic T7 systems, with 10 of the 16 vectors showing enhanced EGFP expression levels compared to the control. These findings indicated that the fore-cistron sequence has a significant effect on the expression of the target gene, which can enhance or weaken their expression. Finally, the four strongest vectors, pET28-HT5, pET28-HT8, pET28-HT12Y, and pET28-HP11Y, were selected, and their EGFP expression levels increased by 68%, 65%, 76%, and 80%, respectively.

To investigate the underlying reason for the increased EGFP expression level in the bicistronic T7 system, we tested the EGFP transcription level of the top four strongest BCD vectors and calculated their translation efficiency. Unlike the generally improved translation efficiency we previously observed in *C. glutamicum* [19], different effects of fore-cistron sequences were observed across the four vectors (Fig. 2B). For example, pET28-HT5-EGFP exhibited increased EGFP expression due to enhanced translation efficiency, while pET28-HP11Y-EGFP showed decreased translation efficiency and improved EGFP fluorescence intensity attributed solely to increased transcriptional level. The reasons behind this deserve further exploration.

### 3.3 The enhanced expression of PCP vaccine proteins Oml1, Oml7, and ApxII

To investigate whether the expression intensity of the four bicistronic T7 vectors will be affected by target gene sequences and assess their efficacy in expressing other recombinant proteins, the four best-performing T7 BCD systems were utilized to express three PCP vaccine antigens Oml1, Oml7, and ApxII. Since the full-length ApxII protein was observed to be expressed as an inclusion body in *E. coli* (data not shown), the truncated ApxII fragment 5 (439-801 aa), which has demonstrated good immunogenicity in previous studies, was selected here for expression [10,11]. As shown in SDS-PAGE, all three antigens were successfully expressed. Moreover, the protein yield per OD\textsubscript{600} of all four BCD vectors exceeded that of the monocistronic control (Fig. 3A-3C). We further calculated the relative protein yield per liter of cells for each vector. The results in Fig. 3D-3F showed that the use of pET28-HT5, pET28-HT8, pET28-HP11Y, and pET28-HT12Y increased the yield of Oml1 by 38%, 29%, 29%, and 24%, the yield of Oml7 by 13%, 8%, 18%, and 17%, and the yield of ApxII by 24%, 18%, 22%, 17%, respectively. These results suggested that all these four bicistronic T7 vectors have good compatibility and can enhance the expression of various proteins. Based on these results, we selected the strongest vector (pET28-HT5-Oml1, pET28-HP11Y-Oml7, and pET28-HT5-ApxII) for each protein for subsequent protein expression experiments.

### 3.4 Optimization of cultivation temperature, induction conditions, and medium components for the production of PCP vaccine proteins in MWP

Apart from the expression element, cultivation temperature, induction conditions, and medium components (such as carbon and nitrogen sources) are also important factors that influence cell growth, cell wall structure, and energy metabolism, thereby affecting protein expression [27–29]. To determine the optimal condition for the production of PCP vaccine proteins, we first investigated the effect of temperature. As shown in Fig. 4A-4C, 25\textdegree C was the most favorable temperature for protein expression among the three temperatures tested, resulting in the highest expression level of Oml1, Oml7, and ApxII. This beneficial effect of lower expression temperature has also been observed in previous studies on the soluble expression of recombinant proteins [30,31]. Therefore, for the subsequent expression of PCP vaccine proteins, the cultivation temperature was set at 25\textdegree C.

We also optimized the IPTG concentration and pre-induction period for PCP antigen expression. Increasing the IPTG concentration from 0 to 0.2 mM resulted in the appearance of Oml1 and Oml7 bands on SDS-PAGE, and ApxII expression was first observed with 0.1 mM IPTG addition (data not shown). No significant effect on cell growth was observed within the IPTG concentration range tested (data not shown). Among the three proteins tested, 0.2 mM IPTG was optimal, and the protein band did not increase with further increases.
in IPTG concentration (Fig. 4D-F). Therefore, 0.2 mM IPTG was used for PCP antigen production. The effect of pre-induction period on protein production was shown in Fig. 4G-4I, where a 2 h pre-induction period was found to be optimal for the expression of Oml1, Oml7, and ApxII. Lower protein expression levels were observed when the pre-induction period was shorter than 2 h. This may be because inducing protein expression during the early growth phase increases the metabolic burden on the cell, ultimately affecting cell growth and protein production. With the further prolongation of pre-induction culture time, the production of the three proteins decreased. Therefore, a 2-h pre-induction period was chosen for subsequent experiments.

Additionally, the effects of different carbon and nitrogen sources on PCP antigen production were also investigated. As shown in Fig. 5A-5C, among all carbon sources tested, glycerol had the most significant positive effect on PCP antigen production. Compared to glucose, the yield of Oml1, Oml7, and ApxII in glycerol increased by 30%, 15%, and 30%, respectively. The effect of nitrogen source on PCP antigen expression was shown in Fig. 5D-5F. Unexpectedly, the extra addition of nitrogen source urea, (NH₄)₂SO₄, and NH₄Cl to TBSB reduced cell growth and protein yield to varying degrees. These findings suggested that the nitrogen source present in the original TBSB is sufficient for both cell growth and protein expression. Thus, in subsequent experiments, the nitrogen source was added according to the original culture medium.

3.5 High-level production of Oml1, Oml7 and ApxII by fed-batch cultivation

To achieve high-level production of Oml1, Oml7, and ApxII in E. coli, fed-batch cultivation was performed for each protein in a 5 L bioreactor with a 2 L working volume. As shown in Fig. 6A-6C, the trend of cell growth and protein accumulation was similar for all three antigens, and the highest cell density and maximum yield were all observed at 30 h after inoculation. The maximum OD600 of Oml1, Oml7, and ApxII were 98, 110, and 80, respectively, and the corresponding protein yield reached 2.43 g/L, 2.59 g/L, and 1.21 g/L. Unlike continuous protein accumulation observed in secretory expression,[12,32] there was a significant correlation between protein accumulation and cell density, protein accumulation ceased when the cell density stopped increasing. Next, the recombinant antigens Oml1, Oml7, and ApxII were purified using a HisTrap HP affinity column. As shown in Fig. 6D-6F, after simple purification steps, all three proteins were successfully purified with high purity (>90%). This allows us to further explore whether these three recombinant antigens are biologically active.

3.6 Protective immunity induced by recombinant PCP antigen against A. pleuropneumoniae

Mice injected intraperitoneally with A. pleuropneumoniae serotype 7 (APP7) were used as protective models to examine the protective immunity of PCP antigens produced here.[5,10] As the 6xHis tag has a small molecular weight and is not charged under physiological conditions, it is speculated that the retention of the 6xHis tag will not affect the immunogenicity of recombinant antigens.[12] As shown in Fig. 6G, Oml1 and ApxII antigen-immunized mice were all well protected against lethal challenge with APP7, 90% and 80% of the mice were still alive even 168 h after infection, respectively. However, unlike the high protective effect of Oml7 previously observed in C. glutamicum,[12] Oml7 showed a relatively low protective effect among the three antigens tested. Only 50% of the mice survived, but the survival rate was still higher than that of the control (20%). This low protection of Oml7 may be due to the damage of protein activity during the purification or storage process. In the challenge experiment with APP7, the good immune protective effect of Oml1 observed here once again proved the good cross-protection effect of the subunit vaccine on different serotypes and its great application prospects for PCP prevention.

4. Discussion

To date, various expression systems have been developed for the production of recombinant proteins, including mammalian cells, insects, plants, yeast, and bacteria.[13,33,34] Among them, the Gram-negative E. coli has long been the “workhorse” of recombinant proteins due to its easy manipulation, rapid growth, clear genetic background, and high-yielding recombinant protein expression, etc.[35,36] For the recombinant expression in E. coli, the expression vector is a key component to determine the protein expression level.[14] Generally, to obtain satisfactory protein yields, expression vectors containing strong promoters are used preferentially. Although numerous strong promoters have been developed for the production of recombinant
proteins over the past decades,[14] the T7 promoter and its derived pET expression vector are still the most widely used and popular commercial expression systems in *E. coli*. In this study, the bicistronic strategy was applied to the T7-based pET28 expression system to further enhance its expression intensity.

Bicistronic expression design, a structure derived from the polycistron in archaea and bacteria, has several advantages over conventional monocistronic expression for recombinant protein production. Due to the prior translation of the fore-cistron, the mRNA secondary structure around the target gene’s translational initiation region (TIR) can be reduced.[20] Moreover, there is translation coupling in bicistronic expression, where the upstream fore-cistron can significantly impact the expression of the downstream target gene.[20,37] This means that a well-translated upstream fore-cistron sequence can be introduced as an “enhancer” to further improve protein yield. This strategy has been applied to improve the expression intensity of tac promoter in *C. glutamicum*.[19] in this study, we further applied it to the T7 expression system. To ensure the successful enhancement of recombinant expression, a series of bicistronic T7 systems containing various fore-cistron sequences were constructed and evaluated. Four of the most effective bicistronic T7 systems were then used to address the low expression status of the PCP subunit vaccine in *E. coli*. As shown in Figure 3, all four BCD vectors exhibited good compatibility and an enhancement effect on the expression of three antigen proteins (Oml1, Oml7, and ApxII). This observed good compatibility can be attributed to the reduction of unfavorable mRNA structures by the translation of fore-cistron, which decreases the impact of the target gene sequence on gene expression level.[20,38] After several rounds of optimization in MWP, the production of Oml1, Oml7, and ApxII in the T7 BCD system gradually increased, and achieved the highest yields reported so far, reaching 2.43 g/L, 2.59 g/L, and 1.21 g/L in a 5-L bioreactor, respectively (Fig. 3). Despite these satisfactory results, there is still much work that can be done. For example, the output of the T7 BCD system still has great room for improvement, the expression intensity of the T7 BCD system can be further improved by optimizing other genetic elements, such as SD2 and the length of fore-cistron. Although the three recombinant antigens produced here induced well-protective immunity against *A. pleuropneumoniae*, the immune protective effect of conjugate antigens Oml1/ApxII, Oml7/ApxII, and Oml1/Oml7/ApxII remains to be evaluated, as previous studies have reported an improved survival rate through combined immunization with Apx toxin and Oml.[4] Additionally, the application of the T7 BCD systems should be extended to the expression of other proteins to further evaluate its expression performance.

In conclusion, this study successfully established a bicistronic T7 expression system for enhancing recombinant protein production in *E. coli*, which led to hyper-production of PCP subunit vaccine proteins Oml1, Oml7, and ApxII. The unprecedented yields of these proteins were achieved through a combination of the T7 BCD system and fermentation condition optimization in MWP, with final yields of 2.43 g/L for Oml1, 2.59 g/L for Oml7, and 1.21 g/L for ApxII in a 5-L bioreactor. This study provided a powerful protein expression system for the enhanced production of recombinant proteins or overexpression of key enzymes in *E. coli*. In addition, it also promoted the development of subunit vaccines against PCP and provided an excellent strain for the production of PCP subunit vaccine proteins.

Supplementary Information

**Supplementary material 1** Gene sequence of Oml1, Oml7, and ApxII. **Table S1** Bacteria strains and plasmids used in this study. **Table S2** Primers used in this study.

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**Author’s contribution**
M.S., X.G, and A.L. performed all experiments and wrote this manuscript with assistance from X. L. and R.L-A.. Z.B. helped revise this manuscript.

Statements and Declarations

Ethics approval All procedures for handling the mice used in this study were reviewed and approved by the Institute of Tecon Biology Co., Ltd (SYXK2021-0006) and performed ethically and humanely.

Conflict of interest The authors declare no competing interests.

Consent for publication All authors approved the consent for publishing the manuscript to Systems Microbiology and Biomanufacturing.

Data availability

All data in this study are included in this published article and its supplementary files.

References


Figure captions

Fig. 1 Construction of bicistronic T7 expression structure
(A) Monocistronic and bicistronic T7 expression structure. The bicistronic T7 expression system contains T7 promoter, 5'UTR and its original SD motif (SD1), fore-cistron sequence, the second SD sequence (SD2), and downstream target gene EGFP. (B) Construction process of bicistronic T7 expression plasmid.

**Fig. 2** Characterization of effects of different fore-cistron sequences on the performance of bicistronic T7 expression system

C (control) represents the monocistronic expression vector pET28-EGFP. The abscissa represents the BCD vector containing the corresponding fore-cistron. (A) EGFP expression level of the BCD expression vectors. (B) The relative transcriptional level and relative translation efficiency of EGFP in bicistronic T7 expression system.

**Fig. 3** Expression level of PCP vaccine antigen in four bicistronic T7 expression vectors.

Line M: Protein marker, Line 1: BL21 WT strain, Line 2: pET28 without target gene, Line 3-7 represented the target protein expression of vector pET28a, pET28-HT5, pET28-HT8, pET28-HP11Y, and pET28-HP12Y, respectively. (A), (B), and (C) represented the SDS-PAGE analysis of Oml1, Oml7, and ApxII, respectively. Approximately 5 μL culture supernatant was loaded onto each lane. (D), (E), and (F) represented the relative protein yield of Oml1, Oml7 and ApxII in BCD vectors per liter of cells, respectively.

**Fig. 4** Effects of cultivation temperature and induction conditions on the production of PCP vaccine proteins.

For the optimization experiment of cultivation temperature, the OD<sub>600</sub> and protein yield at 37 were used as the control, defined as 1. For the optimization experiment of induction conditions, the OD<sub>600</sub> and protein yield with 1.0 mM IPTG addition and 0 h pre-induction period was used as the control, defined as 1. (A) Effect of cultivation temperature on Oml1 production. (B) Effect of cultivation temperature on Oml7 production. (C) Effect of cultivation temperature on ApxII production. (D) Effect of IPTG concentration on Oml1 production. (E) Effect of IPTG concentration on Oml7 production. (F) Effect of IPTG concentration on ApxII production. (G) Effect of pre-induction period on Oml1 production. (H) Effect of pre-induction period on Oml7 production. (I) Effect of pre-induction period on ApxII production.

**Fig. 5** Effects of medium components on the production of PCP vaccine proteins.

For the optimization of carbon source, the OD<sub>600</sub> and protein yield under glucose carbon source were used as the control, defined as 1. For the optimization of nitrogen source, the OD<sub>600</sub> and protein yield in the original TBSB medium were used as the control, defined as 1. (A) Effect of carbon source on Oml1 production. (B) Effect of carbon source on Oml7 production. (C) Effect of carbon source on ApxII production. (D) Effect of nitrogen source on Oml1 production. (E) Effect of nitrogen source on Oml7 production. (F) Effect of nitrogen source on ApxII production.

**Fig. 6** High-level production of Oml1, Oml7, and ApxII in 5 L bioreactor.

(A), (B), and (C) represented the time profile of cell growth, DO level, and protein concentration during fermentation. Arrows represented IPTG addition. (D), (E), and (F) represented SDS-PAGE analysis of purified Oml1, Oml7 and ApxII, respectively. (G) Induction of protective immunity against challenge infection with APP7.
Fig. 1

Monocistronic T7 expression architecture

LacI  T7  5'UTR  SD1  EGFP  Terminator

Bicistronic T7 expression architecture

LacI  T7  5'UTR  SD1  Fore-cistron  SD2  TA  ATG  EGFP  Terminator

Translation coupling frame

Nco I digestion

LacI  T7  5'UTR  SD1  EGFP

Pet28-EGFP

ori

Kan

16 different fore-cistron sequences

62 bp fore-cistron  SD2  TAATG
62 bp fore-cistron  SD2  TAATG
62 bp fore-cistron  SD2  TAATG

homologous recombination

LacI  T7  5'UTR  SD1  62 bp fore-cistron  SD2  TA  ATG  EGFP

ori

Kan

Bicistronic T7 expression vector

Fig. 2

A

Fluorescence intensity (RFU/DD)

0  9000  18000  27000  36000  45000

HT5  HT10  HT15  HT20  HT25  HT30  HT35  HT40  HT45  HT50  HT55

B

Relative translation efficiency

Relative transcription level

HT5  HT10  HT15  HT20  HT25  HT30  HT35  HT40  HT45  HT50  HT55

Fig. 2

A

kDa M  1  2  3  4  5  6  7

B

kDa M  1  2  3  4  5  6  7

C

kDa M  1  2  3  4  5  6  7

D

Relative Omi1 yield

1.50

1.00

0.50

0.00

1  2  3  4  5  6  7

E

Relative Omi7 yield

1.25

0.75

0.25

0.00

1  2  3  4  5  6  7

F

Relative Apa1 yield

1.50

1.00

0.50

0.00

1  2  3  4  5  6  7
Fig. 6