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Fusion expression of *pedA* gene to obtain biologically active pediocin PA-1 in *Escherichia coli**

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Abstract: Two heterologous expression systems using thioredoxin (*trxA*) as a gene fusion part in *Escherichia coli* were developed to produce recombinant pediocin PA-1. Pediocin PA-1 structural gene *pedA* was isolated from *Pediococcus acidilactici* PA003 by the method of polymerase chain reaction (PCR), then cloned into vector pET32a(+), and expressed as thioredoxin-PedA fusion protein in the host strain *E. coli* BL21 (DE3). The fusion protein was in the form of inclusion body and was refolded before purification by nickel-iminodiacetic acid (Ni-IDA) agarose resin column. Biological activity of recombinant pediocin PA-1 was analyzed after cleavage of the fusion protein by enterokinase. Agar diffusion test revealed that 512-arbitrary unit (AU) recombinant pediocin PA-1 was obtained from 1 ml culture medium of *E. coli* (pPA003PED1) using *Listeria monocytogenes* as the indicator strain. Thioredoxin-PedA fusion gene was further cloned into pET20b(+). Thioredoxin-PedA fusion protein was detected in both the periplasmic and cytoplasmic spaces. The recombinant pediocin PA-1 from the soluble fraction attained 384 AU from 1 ml culture medium of *E. coli* (pPA003PED2). Therefore, biologically active pediocin PA-1 could be obtained by these two hybrid gene expression methods.

Key words: Bacteriocin, Fusion expression, Inclusion body, Pediocin PA-1, Thioredoxin
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1 Introduction

In the food industry, food preservation has always been an important aspect. Searching new types of biological preservatives with low toxicity and high efficiency has been a strong focus in recent years. Bacteriocins produced by lactic acid bacteria (LAB) have gained particular attention owing to their potential application as the substitute of artificial chemical preservatives.

Bacteriocins of LAB are ribosomally synthesized proteins which have long been used in the fermentation and preservation of meat and milk (Gillor *et al*., 2008). Pediocin PA-1 belongs to the Class IIa bacteriocins and gained commercial interest owing to its characteristics of heat stability, high specific activity against the foodborne pathogen *Listeria monocytogenes*, and no effect on the main composition of the mouse intestinal ecosystem (Kheadr *et al*., 2010). However, production of pediocin PA-1 using bacteriocin-producing LAB involves low fermentation yields and complex processes in the purification of bacteriocins (Yildirim *et al*., 2007). Heterologous expression in *Escherichia coli* systems is currently utilized to produce bacteriocins since *E. coli* strains have relatively clear genetic backgrounds which are convenient to control the gene expression and attain low culture costs with higher production of interest proteins. However, there is no guarantee of obtaining biologically active heterologous protein, as high levels of expression sometimes cause misfolding and aggregation (Austin, 2003).

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We reported the cloning of the pediocin PA-1 structural gene *pedA* from *Pediococcus acidilactici* PA003 (Zhou *et al*., 2006) into the *E. coli* vector pET32a(+) and induced expression of the thioredoxin-PedA fusion protein. Recombinant protein was overexpressed as an inclusion body. Separation, renaturation, and enterokinase cleavage processes have been investigated, resulting in the production of biologically active pediocin PA-1. We further studied fusion expression of thioredoxin-PedA in pET20b(+), a potential periplasmic expression vector with a *pelB* signal sequence. Biologically active pediocin PA-1 was finally obtained in this system as a soluble fraction.

2 Materials and methods

2.1 Bacterial strains, plasmids, and culture conditions

P. acidilactici PA003, a wild pediocin PA-1 producing strain, was grown in MRS medium. *E. coli* DH5α and *E. coli* BL21 (DE3) were grown in Luria-Bertani (LB) medium. Transformants of *E. coli* were cultivated in LB medium supplemented with ampicillin (100 μg/ml). The indicator strain used in the bacteriocin assay was *L. monocytogenes* CVCC1595 (China Institute of Veterinary Drug Control), which was grown in broth medium. Plasmid pMD18-T, pET32a(+), and pET20b(+) were applied to the constructions of thioredoxin-PedA hybrid gene.

2.2 Cloning of the *pedA* gene and constructions of plasmids

All DNA manipulations were carried out according to the procedures described by Sambrook and Russell (2001). Total DNA of *P. acidilactici* PA003 was used as the template to amplify the *pedA* gene. The forward primer PedA1F (5′-AACCCCAGATCT CGACGACGACGACAAGAAATACTACGGTAA TGGG-3′) contains a restriction site for *Bgl*II (AGATCT) and codons (GACGACGACGACAAG) for 5 amino acids (-Asp-Asp-Asp-Asp-Lys-) that are the enterokinase cleavage sequence. The reverse primer PedA1R (5′-CCCGGGCTCGAGTTATGAT GCCAGCTCAGCATAATGCTA-3′) contains *Xho*I restriction enzyme site (CTCGAG). The polymerase chain reaction (PCR) amplification was performed as follows: initial denaturation of DNA at 94 °C for 3 min, an amplification programme consisting of 30 cycles of 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 60 s, and the final extension of 1 cycle at 72 °C for another 7 min. The PCR product was purified and ligated to pMD18-T vector. The T-A cloning product was digested with *Bgl*II and *Xho*I restriction endonucleases. The resulted *pedA* gene fragment was purified and ligated into pET32a(+) vector digested with the same restriction enzymes in order to be cloned in frame with thioredoxin and $(His)_6$ genes. Ligation mixture was transformed into *E. coli* DH5α competent cells. The recombinant plasmid confirmed by DNA sequencing was further used to transform *E. coli* BL21 (DE3) for thioredoxin-PedA fusion protein expression and the resulting recombinant plasmid was designated pPA003PED1 (Fig. 1).

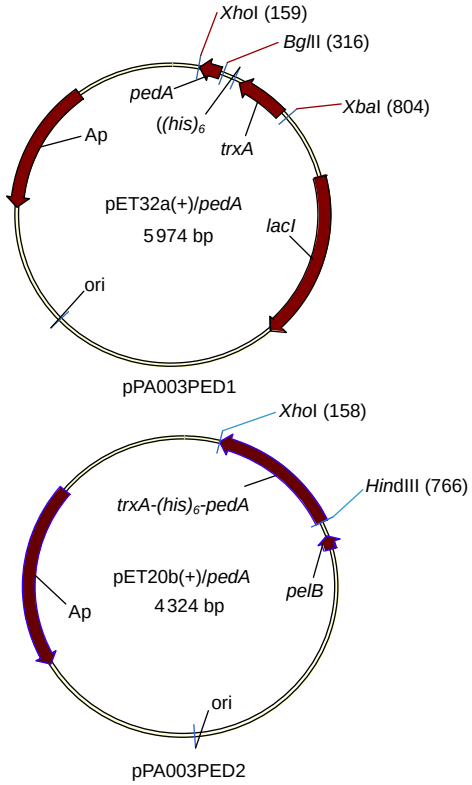

Fig. 1 Circular maps of constructions of pPA003PED1 (pET32a(+) harboring *pedA* gene) and pPA003PED2 (pET20b(+) harboring thioredoxin-PedA fusion gene) with open reading frames (ORFs) and antibiotic markers
Relevant restriction enzyme sites are indicated

Recombinant pPA003PED1 was used as the template to amplify the thioredoxin-PedA fusion gene

by primer PedA2F (5′-AACCCCAAGCTTATGAG CGATAAAATTATTCACCTGACTGACG-3′) and PedA2R (5′-CCCGGGCTCGAGCTATTATCAGC ATTTATGATTACCTTGATGTCCA-3′) under the same programme. After digestion of pET20b(+) and PCR product with the endonucleases *Hin*dIII and *Xho*I, the fragment was subcloned into the *Hin*dIII-*Xho*I sites of the cassette with the resulting vector being named pPA003PED2 (Fig. 1).

2.3 Expression of thioredoxin-PedA gene in *E. coli*

For protein expression, 1 ml of overnight culture of *E. coli* BL21 (DE3) harboring recombinant plasmid was inoculated into 100 ml LB broth containing ampicillin (100 μg/ml) and incubated at 37 °C with agitation. The culture was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) (20 μmol/L of final concentration both for pPA003PED1 and pPA003PED2) when the optical density at 600 nm (OD_{600}) reached 0.6, and further cultivated under the same conditions. The cells were harvested after 4-h induction.

2.4 Cellular localization of the expressed thioredoxin-PedA

To determine the subcellular fractionation, the culture of *E. coli* (pPA003PED1) was centrifuged for 10 min at 8000×*g*. Cells were resuspended in lysis buffer (50 mmol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl). The mixture was sonicated 50 times for 10 s (200 Hz) with pauses on ice for 20 s. The pellet was collected by centrifugation (8000×*g*, 10 min, 4 °C) and further dissolved in 8 mol/L urea as an insoluble cytoplasmic fraction. The supernatant was kept as the soluble cytoplasmic fraction.

The culture of *E. coli* (pPA003PED2) was centrifuged for 10 min at 8000×*g* and the supernatant was kept as the extracellular fraction. The cells were resuspended and separated into different fractions based on the method of Sommer *et al*. (2010). The final cell debris after sonication was dissolved in 8 mol/L urea as the insoluble cytoplasmic fraction.

2.5 Renaturation of inclusion body and purification of thioredoxin-PedA

The harvested cell pellets (pPA003PED1) were washed twice by lysis buffer and then resuspended in 5 ml lysis buffer. The mixture was sonicated 50 times for 10 s (200 Hz) with pauses of 20 s on ice, and then centrifuged at 3000×*g* for 20 min. The pellets were washed three times by wash buffer (50 mmol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl, 2 mol/L urea, 1% (v/v) Triton X-100) to obtain the clean inclusion body. The inclusion bodies were further solubilized in two kinds of solubilisation buffer (solubilisation buffer A: 50 mmol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl, 8 mol/L urea, 0.2 mmol/L oxidized glutathione, 2 mmol/L reduced glutathione, 1 mmol/L ethylenediaminetetraacetic acid (EDTA) (pH 8.0), and 0.1 mol/L phenylmethanesulfonyl fluoride (PMSF); solubilisation buffer B: solubilisation buffer A added with 50 mmol/L β-mercaptoethanol) by incubation at 4 °C overnight with constant mixing. Then the soluble portion was diluted 15-fold in renaturing buffer (50 mmol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl, 0.2 mmol/L oxidized glutathione, 2 mmol/L reduced glutathione, 1 mmol/L EDTA (pH 8.0), 5% (v/v) glycerol) slowly with mild agitation. The supernatant was dialyzed against the lysis buffer by 3.5-kDa (cut-off) regenerated cellulose membrane for 24 h and then filtered through a 0.45-μm filter membrane before loaded onto nickel-iminodiacetic acid (Ni-IDA) His-bind resin column (Weishibohui Science and Technology Co., Ltd., Beijing, China). After washing with 100 ml of lysis buffer containing 50 mmol/L imidazole, the fusion protein was eluted with lysis buffer containing 400 mmol/L imidazole. Elutes were collected for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

2.6 Treatment of enterokinase and recovery of biologically active pediocin PA-1

Eluted fusion protein from Ni-IDA column was dialyzed against a cleavage buffer (20 mmol/L Tris-HCl (pH 7.4), 50 mmol/L NaCl, 2.5 mmol/L $CaCl_2$) by 3.5-kDa (cut-off) dialysis bag for 48 h, and then treated overnight with recombinant enterokinase (Majorbio Biotech Co., Ltd., Shanghai, China) at 23 °C. The liberated pediocin PA-1 was purified by Ni-IDA column in which the fusion part and enterokinase containing the His tag will bind to the Ni column. Agar diffusion test was used to determine the biological activity of the recombinant product. The prepared agar plate was overlaid with 10 ml of soft agar inoculated with 50 μl of an overnight culture of *L. monocytogenes* CVCC1595. A total of 12.5 μl samples were added to

a well and then in twofold serial dilutions. Plates were incubated at 37 °C for 16 h. One arbitrary unit (AU) was defined as the reciprocal of the highest dilution yielding a visible inhibition zone. The total AUs per milliliter were calculated by 80×2^n, where n is the number of wells showing inhibition.

3 Results

3.1 Expression of thioredoxin-PedA gene in *E. coli*

Two constructed plasmids (pPA003PED1 and pPA003PED2) were verified by DNA sequencing. The nucleotide sequence homology of *pedA* gene obtained from *P. acidilactici* PA003 showed 100% identity compared with the *pedA* gene that has been published in the National Center for Biotechnology Information (GenBank No. AY083244). The expressed proteins were investigated by SDS-PAGE (Fig. 2). New protein bands were clearly testified around 20 kDa, which corresponded with the predicted molecular mass of recombinant proteins. When the incubation temperature was changed to 25, 30, or 32 °C, they could also be produced (data not shown). Therefore, the fusion proteins were stably expressed in a wide range of conditions. It is worthwhile to note that a low concentration of IPTG (20 μmol/L) worked well in these two systems, revealing cost-saving property.

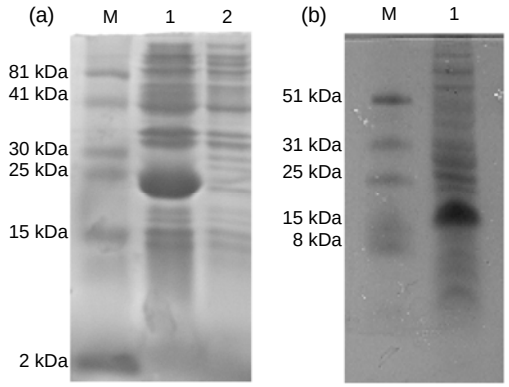

Fig. 2 SDS-PAGE analysis of the expressed proteins in recombinant *E. coli* BL21 (DE3)
(a) *E. coli* (pPA003PED1). Lane M: polypeptide molecular mass standards; Lane 1: whole-cell lysate with IPTG induction (20 μmol/L); Lane 2: whole-cell lysate without IPTG induction; (b) *E. coli* (pPA003PED2). Lane M: polypeptide molecular mass standards; Lane 1: whole-cell lysate with IPTG induction (20 μmol/L)

3.2 Cellular localization of the expressed thioredoxin-PedA

The fact that the target protein was present in the pellet rather than the supernatant fluid after ultrasonic disruption of *E. coli* (pPA003PED1) indicated that His-tagged fusion pediocin PA-1 was expressed as an inclusion body (Fig. 3a). Although low temperature and low concentration of IPTG have been tested, the form of recombinant protein in *E. coli* (pPA003PED1) has not changed probably because of high yield. When produced in pET20b(+), a vector carrying *pelB* signal peptide and having the potential of expressing protein in the periplasmic space, the fusion protein turned into soluble form. Fig. 3b illustrated that thioredoxin-PedA was maintained basically in the cytoplasmic and periplasmic spaces as the soluble fraction. Comparison of the periplasmic and cytoplasmic protein patterns shows that *E. coli* (pPA003PED2) kept a detectable amount of thioredoxin-PedA in the periplasm and more fusion proteins were obtained in the cytoplasm. The fewer protein bands displayed in SDS-PAGE suggested a relatively low amount of host proteins in the periplasmic space, which could provide a simpler purification process.

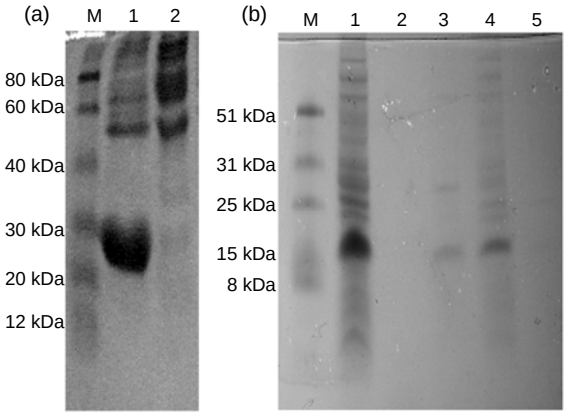

Fig. 3 SDS-PAGE analysis of proteins isolated from different fractions of recombinant strains after induction
(a) *E. coli* (pPA003PED1). Lane M: polypeptide molecular mass standards; Lane 1: proteins in the precipitation after ultrasonic cell disruption; Lane 2: proteins in the supernatant after ultrasonic cell disruption; (b) *E. coli* (pPA003PED2). Lane M: polypeptide molecular mass standards; Lane 1: whole-cell lysate; Lane 2: supernatant proteins; Lane 3: periplasmic proteins; Lane 4: cellular soluble proteins; Lane 5: cellular insoluble proteins

3.3 Renaturation of inclusion body and purification of thioredoxin-PedA

Renaturation process was performed slowly using reduced glutathione (GSH) and oxidized glutathione (GSSH) that provided the appropriate redox potential for both the formation and reshuffling of disulphide bonds. After the renaturation process, target fusion peptide was highly purified by Ni column with the concentration of 0.6 mg/ml appropriately (Fig. 4a). Finally, both the refolded fusion protein (produced in *E. coli* (pPA003PED1)) and the natural fusion protein (produced in *E. coli* (pPA003PED2)) were successfully purified in the range of 30–50 mg/L of bacteria culture. As shown in Fig. 4, two proteins were obtained after enterokinase cleavage and recombinant pediocin PA-1 could flow through Ni column while thioredoxin will bind to the resin because of His tag.

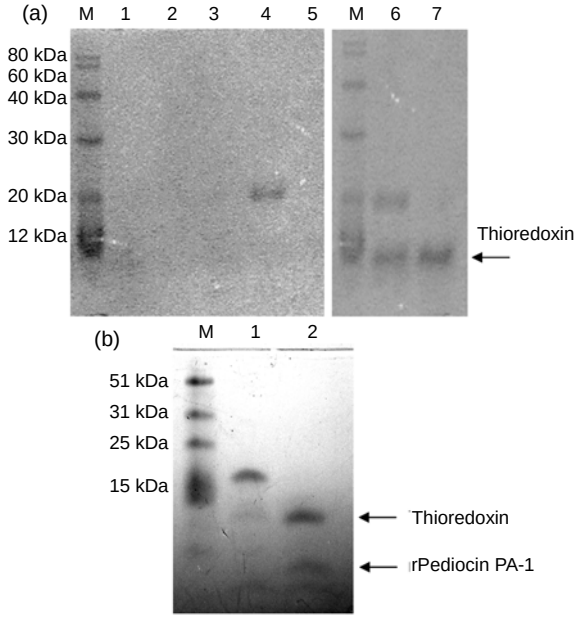

Fig. 4 SDS-PAGE analysis of the proteins resulting from purification and enterokinase cleavage
(a) Purification. Lane M: polypeptide molecular mass standards; Lanes 1–5: elutes of refolded thioredoxin-PedA fusion protein from the Ni-IDA column with lysis buffer containing 400 mmol/L imidazole; Lane 6: elute of partial enterokinase cleavage products from the Ni-IDA column with lysis buffer containing 400 mmol/L imidazole; Lane 7: elute of complete enterokinase cleavage products from the Ni-IDA column with lysis buffer containing 400 mmol/L imidazole; (b) Enterokinase cleavage. Lane M: polypeptide molecular mass standards; Lane 1: thioredoxin-PedA fusion protein eluted from the Ni-IDA column with lysis buffer containing 400 mmol/L imidazole; Lane 2: proteins after enterokinase cleavage

3.4 Biological activity of recombinant pediocin PA-1

Biologically active recombinant pediocin PA-1 was obtained and verified by agar diffusion assay. We adopted two kinds of buffers for solubilisation of inclusion body which resulted in 640 and 10240 AU/ml antibacterial activities, respectively, as illustrated in Fig. 5. Therefore, the addition of β-mercaptoethanol will facilitate the opening of wrong disulfide bonds and the formation of active conformation. While the soluble recombinant pediocin PA-1 in pET20b(+) system revealed 5120 AU/ml antibacterial activity in Fig. 6. Finally, 512-AU recombinant pediocin PA-1 was obtained from 1 ml culture medium of *E. coli* (pPA003PED1) and 384-AU from that of *E. coli* (pPA003PED2).

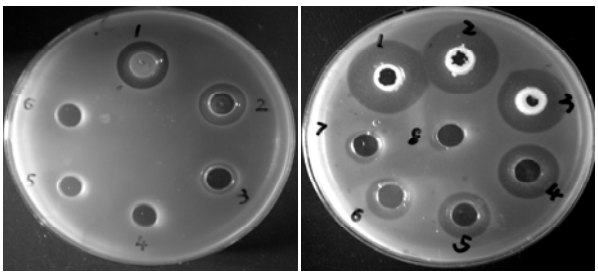

Fig. 5 Agar diffusion assay of recombinant pediocin PA-1 produced by *E. coli* (pPA003PED1)
Biological activity of recombinant pediocin PA-1 product after treatment with solubilisation buffer A (n=3) (a) and solubilisation buffer B (n=7) (b)

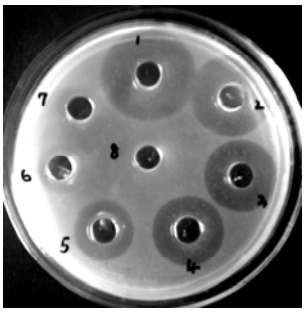

Fig. 6 Agar diffusion assay of recombinant pediocin PA-1 produced in *E. coli* (pPA003PED2) (n=6)

4 Discussion

Fusion expression has been used to increase expression levels and decrease toxicity, as well as to provide purification tags in heterologous expression

strategy (Ingham and Moore, 2007). In particular, thioredoxin gene fusion system is one of the four most successful systems in producing correctly folded and soluble heterologous protein in *E. coli* (Fu *et al*., 2005). Thioredoxin was chosen as the fusion partner since the pediocin PA-1 is a small peptide and is liable to protease hydrolysis, and thioredoxin may promote the formation of the correct structure of the target fusion protein (Austin, 2003). It is known that most of the recombinant antimicrobial peptides have been expressed in *E. coli* as inclusion bodies to protect the host cells from the toxic effects of the recombinant peptides (Lee *et al*., 2000) and effectively remove the impurity of many sorts of soluble proteins. Austin (2003) and Tian *et al*. (2007) have constructed recombinants in pET32b and expressed thioredoxin-fused proteins in *E. coli* BL21 (DE3) mainly as inclusion bodies. It is possible that recombinant proteins are liable to be overexpressed and aggregated in this expression system. Therefore, we tried to explore whether pediocin PA-1 would be fusion-expressed as inclusion body and recovered biological activity in pET32a(+) system. The result coincided with our assumption and provided another way of producing recombinant pediocin PA-1. As well, the refolding protocol could offer a reference for significant protein renaturation.

The previous research conducted by Beaulieu *et al*. (2007) produced thioredoxin-fused pediocin PA-1 in *E. coli* Origami (DE3). About 20–30 mg/L recombinant pediocin PA-1 was gained, which corresponded to an increase of 4-fold to 5-fold in comparison with the pediocin PA-1 yields obtained with their naturally producing strain. In contrast to their work, we chose *E. coli* BL21 (DE3) as the host strain and adopted AU/ml as the measuring standard since pediocin PA-1 is an antimicrobial peptide. Compared with the naturally producing strain *P. acidilactici* PA003, the pediocin PA-1 yield of *E. coli* (pPA003PED1) increased from 6.2 to 512 AU/ml.

Furthermore, we assayed the possibility of pET20b(+) to produce recombinant pediocin PA-1 in a different way since *pelB* signal peptide was designed to transport target proteins into periplasmic space, which provides a more oxidative environment and less protease activity than the cytoplasm (Makrides, 1996). Although there is no general rule in selecting a proper signal sequence for a certain recombinant protein to guarantee its successful secretion (Choi and Lee, 2004), our results proved the fact that thioredoxin-PedA was secreted into the periplasmic space partially. Consequently, stress from high concentration of recombinant protein in cytoplasm was relieved and a soluble form was realized.

In this study, we tested two hybrid gene expression methods which could produce biologically active pediocin PA-1. More researches are needed to optimize the protocol for purification and renaturation processes in order to maximize the active pediocin PA-1 output. Although the purpose of pediocin PA-1 expression and purification was laboratory use, our strategy may provide a solution for low pediocin PA-1 production in the wild strain.

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