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Human neutrophil peptide 3 could be functionally expressed in *Rhodobacter sphaeroides*

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Human neutrophil peptides (HNPs) possess high antimicrobial activities against a broad spectrum of microorganisms. *Rhodobacter sphaeroides* is the best-characterized photosynthetic bacterium and exhibits potential as a novel expression system. Up to date, no literature has been reported regarding expression of HNP3 in *Rb. sphaeroides*. In the present study, the *HNP3* gene fragment was amplified by SOE PCR and ligated into photosynthetic bacteria light-harvesting complex 2 (LH2) expression vector leading to HNP3 fusion protein expression vector. The HNP3 fusion protein was successfully expressed as rapidly evaluated by the LH2 characteristic peaks at ~800 nm and ~850 nm before purification and SDS/PAGE. Subsequently, the HNP3 fusion protein was purified by one-step affinity chromatography, and could be rapidly detected by the color and the spectral absorption at ~800 nm and ~850 nm before SDS/PAGE. Antimicrobial activity assay suggested that the HNP3 fusion protein exhibited high antimicrobial activity towards *E. coli*. The present study may supply an insight into employing the novel *Rb. sphaeroides* expression system, exhibiting dramatic advantages over currently used commercial expression system, to heterologously express human neutrophil peptides.

Key words: *Rb. sphaeroides*, HNP3, expression system, antimicrobial activity, spectral absorption

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INTRODUCTION

Rhodobacter sphaeroides is one of the best-characterized photosynthetic bacteria and has been employed as an excellent model system for studying membrane development (Chi *et al*., 2014; Kiley & Kaplan, 1987). The model purple bacterium can be grown under aerobic and anaerobic respiration, fermentation and anoxygenic photosynthesis growth conditions. Under low oxygen and optimal light intensity, photosynthetic apparatus of this bacterium will be largely formed in the intracytoplasmic membrane system (Adams & Hunter, 2012; Niederman, 2013; Pemberton *et al*., 1998; Woronowicz *et al*., 2013). The photosynthetic apparatus mainly is comprised of light-harvesting complex 1, LH2 and reaction center, which play crucial roles in the photosynthesis and the survival of this bacterium (Hu *et al*., 2002; Tucker *et al*., 2010). The LH2 complexes have two characteristic absorption bands at ~800 nm and ~850 nm. The higher spectral absorption peak at ~800 nm and ~850 nm indicates a greater amount of LH2. Consequently, the characteristic absorption could be used to evaluate the production of LH2 very rapidly and conveniently. Based on this theory, we primarily constructed a novel *Rb. sphaeroides* expression system to rapidly evaluate heterologously expressed protein levels (Zhao *et al*., 2011).

Human neutrophil peptide (HNP) with the size of about 3.5 kDa is present in a wide range of species and is comprised of 29 to 35 amino acids (Spencer *et al*., 2004). It has been well characterized that HNPs possess high antimicrobial activities against Gram-negative and Gram-positive bacteria, fungi and viruses (Hartshorn *et al*., 2006; Kagan *et al*., 1994). Moreover, HNPs exert immune-modulating effects by activation of costimulatory molecules in lung epithelial cells and CD4+ lymphocytes (Vaschetto *et al*., 2007). It has been suggested that HNPs might be developed as a new drug to treat infections (Hancock, 1997). HNP3 has been reported to increase the production of proinflammatory cytokines (TNF and IL-1) (Chaly *et al*., 2000) and is proposed to serve as a tumour biomarker (Albrethsen *et al*., 2005). On the other hand, HNP3 expression level was found to be elevated in the plasma and tumour tissue of patients with colorectal cancer (Albrethsen *et al*., 2006; Melle *et al*., 2005). Consequently, HNP3 may play important roles in some cancer treatment.

Isolation from a host organisms and chemical synthesis are two traditional ways to obtain HNP3. However, isolation from host organism requires large amounts of materials and there is a problem of having very low yields. Chemical synthesis generates high costs and low yields. Heterologous expression in *E. coli* is by far the simplest approaches to produce large amounts of HNP3. However, due to its toxicity to host cells, it is difficult to obtain high level expression of HNP3 in *E. coli*. *Rb. sphaeride* is the best-characterized photosynthetic bacterium and exhibits potential as a novel expression system as described in our previous study. Up to date, literatures regarding HNP3 exerting antimicrobial activity against *Rb. sphaeroides* and heterologous expression of HNP3 in *Rb. sphaerides* have not been reported. In the present study, we amplified the *HNP3* gene fragment by SOE PCR and expressed it in the novel *Rb. sphaeroides* expression system. Our present study may supply a novel expression system for heterologous expression of human neutrophil peptides.

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Table 1. Primer sequences used for amplification of *HNP3*

primer	Sequence (5′-3′)
P1	5′-GAGTACTGATTGTTATTGTCGTATTCCTGCTT-3′
P2	5′-GTACCATAACGACGTTCACCAGCAATACAAGCAGGAATACGACAA-3′
P3	5′-ACGTCGTTATGGTACTTGTATTTATCAAGGTCGTCTTTGGGC-3′
P4	5′-GCTCTAGAACAACAAAAAGCCCAAAGACGACCTT-3′

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Rb. sphaeroides* strains were grown at 34°C in M22+ medium supplemented with 0.1% casamino acids for growth in liquid culture (Hunter & Turner, 1988). *E. coli* strains were grown aerobically at 37°C in LB medium. Antibiotics were added to the growth media at the following concentrations: 100 μg/ml ampicillin and 10 μg/ml tetracycline for *E. coli*, and 1 μg/ml tetracycline, 20 μg/ml kanamycin, 5 μg/ml streptomycin, and 30 μg/ml gentamycin for *Rb. sphaeroides*.

Construction of *HNP3* expression vectors. *HNP3* gene fragment was amplified by SOE PCR using the primers listed in Table 1. All the primer sequences were designed according to the reported HNP3 amino acid sequence (Raj *et al*., 2000). Two rounds of PCR were performed to obtain the *HNP3* gene fragment. In the first round PCR, *P1P2*, *P3P4* fragments were amplified with primers P1–P2 and P3–P4, respectively. In the second round PCR, *HNP3* fragment was obtained with primers P1–P4 by using the *P1P2* and *P3P4* fragments as template. The final PCR product was cloned into pMD18-T cloning vector and sequenced. Then the target gene was cut from pMD18-T-*HNP3* by *Sac*I and *Xba*I, and ligated into the pRK*puc*P*lacI*^{q}*puc1B*$_{His10}$*1AC* vector digested with the same restriction enzymes, producing the expression vector pRK*puc*P*lacI*^{q}*puc1B-HNP3*$_{His10}$*-1AC*. The recombinant was identified by restriction enzyme analysis and sequencing (BGI, China).

Expression of HNP3 in *Rb. sphaeroides*. Plasmid DNA was mobilized into *Rb. sphaeroides* CQU68 mutant strain (genomic deletion of *pufBALMX*, *puc1BA* and *puc-2BA*) by using *E. coli* S17-1 as the donor, as described previously (Hunter & Turner, 1988). Transconjugants were grown aerobically in the dark on M22+ medium plates supplemented with appropriate antibiotics as described above. For the expression of HNP3 fusion protein, cell cultures were shifted from aerobic conditions to micro-aerobic conditions at an OD_{600} of 0.5–1.0, and incubated at 34°C for 8 hours after the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM. Spectral absorptions were recorded on a lambda 900 UV/VIS Spectrometer (Perkin-Elmer).

One-step purification of HNP3 fusion protein from *Rb. sphaeroides*. Crude membranes were isolated from *Rb. sphaeroides* as described in our previous report (Zhao *et al*., 2010). HNP3 fusion protein was purified by affinity binding on Ni-IDA agarose resin (Amersham). The affinity column was washed with 10 column volumes of buffer A (20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 0.1% (v/v) *N*,*N*-dimethyldodecylamine-*N*-oxide) containing 10 mM imidazole. To decrease the background of unspecific binding, an additional washing with 10 column volumes of buffer A containing 30 mM imidazole was performed. HNP3 fusion protein was eluted from the column with buffer A containing 250 mM imidazole. Then, the purified protein was separated on 15–20% gradient SDS-PAGE and transferred to PVDF membrane. Anti-His antibody was used as the primary anti-body at a 1:1500 dilution in PBST with 5% dry milk. Immunoblots were visualized using D-AB and recorded on a GS-800 Calibrated Densitometer (Bio-Rad).

Antimicrobial activity assay. The antimicrobial activity assay was performed with Oxford cup method. *E. coli* DH5α was used as the indicator strain. A single colony of the DH5α was inoculated into 10 mL of LB medium and shaken overnight at 37°C. 10 mL of LB medium were then inoculated with 100 μL of the preculture and shaken at 37°C until OD600 was equal to 0.3–0.5. Cell cultures were used as an indicator strain. To make the plate, LB agar (1.5%) medium was first placed in plates. Then 100 μL of DH5α were added to 100 mL of the LB agar (0.7%) medium and subsequently overlaid on the LB agar (1.5%) medium. Wells were prepared by Oxford cup. In each well, 25 μL of 50 mg/mL kanamycin and the purified HNP3 fusion protein was added respectively and cultured at 37ºC for about 8 h. The diameter of the inhibition zone extending laterally around the well was measured by a vernier caliper. The experiment was repeated 3 times.

RESULTS AND DISCUSSION

Construction of the *HNP3* expression vector

The *HNP3* fragment was amplified by SOE PCR and ligated into pMD18-T cloning vector and sequenced. After sequencing verification, the *HNP3* gene fragment was cut by *Sac*I and *Xba*I and then ligated into the previously constructed *Rb. sphaeroides* expression vector pRK*puc*P*lacI*^{q}*puc1B*$_{His10}$*1AC* (Hu *et al*., 2010), resulting in the *HNP3* expression vector pRK*puc*P*lacI*^{q}*puc1B-HNP3*$_{His10}$*-1AC*, as shown in Fig. 2. Obviously, the *HNP3* gene was fused to LH2 β-polypeptide. The HNP3 peptide could be assembled into the introcytoplasmic membrane system with LH2 β-polypeptide by PucC protein, which plays very important roles in the formation of LH2 in *Rb. sphaeroides* (Jaschke *et al*., 2008).

Expression of the HNP3 fusion protein

As expected, the HNP3 fusion protein was expressed in *Rb. sphaeroides* CQU mutant in micro-aerobic growth

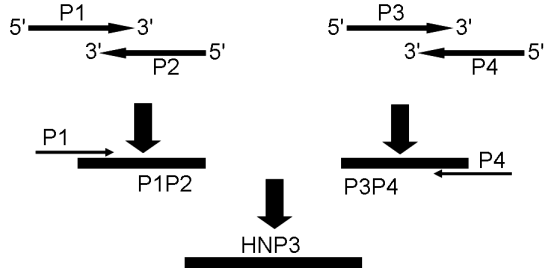

Figure 1. Schematic representation of *HNP3* production by SOE PCR.
Target gene *HNP3* was synthesized by two rounds of PCR with 4 primers designed according to the reported HNP3 amino acid sequence.

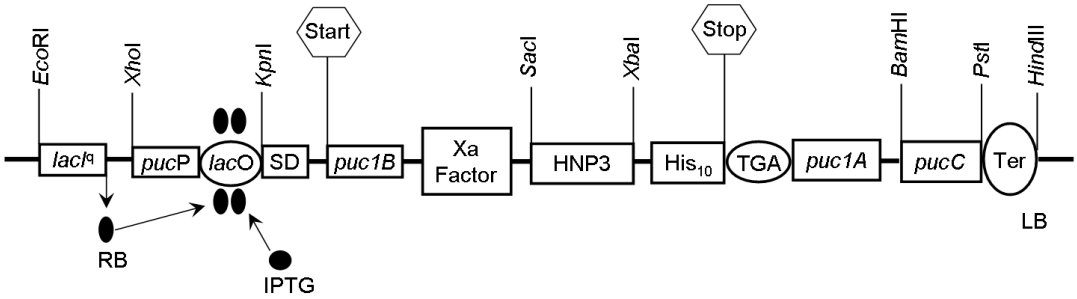

Figure 2. Structure of *HNP3* expression vector pRK*pucP*lacI^q*puc1B-HNP3*$_{His10}$*1AC*.
The vector contains a hybrid promoter comprised of *E. coli lacI*^{q} and lacO, *puc* promoter; gene expression is tightly regulated by IPTG and oxygen tension. The *HNP3* gene was fused to *puc1B* encoding LH2 β-polypeptide. The Xa factor will be used to remove LH2 β-polypeptide from the fusion protein and produce pure HNP3.

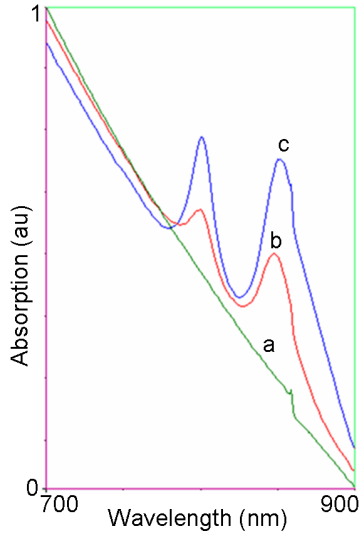

Figure 3. Spectral absorbance of cell cultures expressing LH2 and LH2 with β-polypeptide-HNP3 fusion protein.
a, **b** and **c** indicate cell cultures of *Rb. sphaeroides* CQU68, *Rb. sphaeroides* CQU68/pRK*pucP*lacI^q*puc1B-HNP3*$_{His10}$*-1AC* and *Rb. sphaeroides* CQU68/pRK*pucP*lacI^q*puc1B*$_{His10}$*1AC*, respectively.

conditions, as shown in Fig. 3. Two spectral bands were observed at approximately 800 nm and 850 nm, suggesting the successful expression of the HNP3 fusion protein. *Rb. sphaeroides* CQU68 was mutated with the genomic deletion of *puf* operon and *puc* operons. Thus, no bands were produced at approximately 800 nm and 850 nm, as indicated by *a* in Fig. 3, which was in agreement with previous study (Burgess *et al.*, 1989). *b* and *c* represent *Rb. sphaeroides* CQU68 harboring the plasmid encoding the LH2 and LH2 with β-polypeptide HNP3 fusion protein. Clearly, two bands were observed at approximately 800 nm and 850 nm.

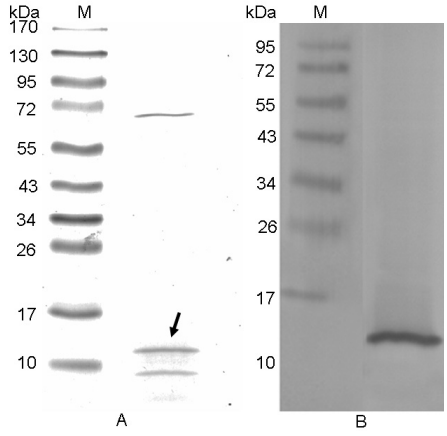

Figure 4. SDS/PAGE (A) and western blot (B) analysis of purified LH2 β-polypeptide-HNP3 fusion protein.

Moreover, the HNP3 was integrated into the intracytoplasmic membrane accompanied with LH2 β-polypeptide. For further demonstration of the HNP3 fusion protein expression, it was purified from *Rb. sphaeroides* and checked by SDS/PAGE and western blot, as indicated in Fig. 4A and 4B. There were two close neighboring protein bands at the size of about 10 kDa. High-resolution three-dimensional crystal structures of LH2 has demonstrated that LH2 from *Rb. sphaeroides* was comprised of nine heterologous α/β-polypeptides (Walz *et al.*, 1998). The nine α-polypeptides could closely interact with the nine β-polypeptides and thus the α-polypeptides could be one-step purified with β-polypeptides-HNP3 fusion protein (Zhao, *et al.*, 2010). On the other hand, the elute was pink because of the existence of the LH2, which in turn indicated the presence of the HNP3 fusion protein. Unspecific band with the size of approximately 70 kDa was observed and it will be removed by ultra-filtration technology in the future. For further experiments, pure HNP3 will be obtained by removing the LH2 β-polypeptides through the factor Xa. Optimization of the HNP3 fusion protein expression levels by vary-

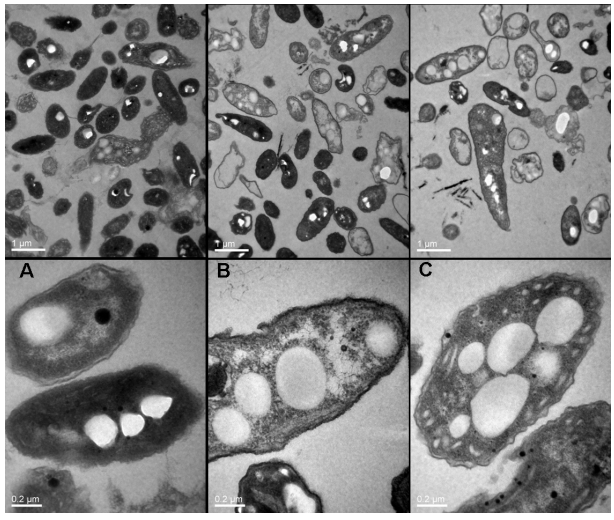

Figure 5. Electron micrographs of cell cultures.
A, **B** and **C** indicate cell cultures of *Rb. sphaeroides* CQU68, *Rb. sphaeroides* CQU68/pRK*pucP*lacI^q*puc1B*$_{His10}$*1AC* and *Rb. sphaeroides* CQU68/pRK*pucP*lacI^q*puc1B- HNP3*$_{His10}$*-1AC*, respectively.

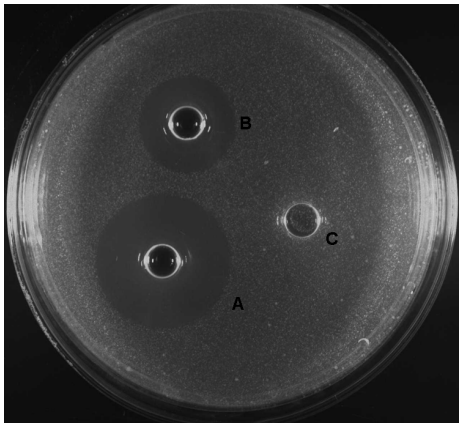

Figure 6. Antimicrobial activity assay of HNP3 fusion protein. **A**, **B** and **C** indicate kanamycin, HNP3 fusion protein and elution buffer, respectively.

ing IPTG concentrations and expression temperature is needed as a next step. On the other hand, expression of the HNP3 fusion protein under light growth conditions will be done in the future since the photosynthesis genes *puc1B, puc1A* and *pucC* can be largely synthesized under light conditions (Pemberton *et al*., 1998) and thus the HNP3 protein will be largely expressed simultaneously.

To detect whether the fusion protein induced any effects on the *Rb. sphaeroides* cells, electron micrographs of thin sections *Rb. sphaeroides* mutant and *Rb. sphaeroides* mutant cells expressing the LH2 and LH2 with β-ploypeptides-HNP3 fusion protein were taken, as seen in Fig. 5. Obviously, all the cells retained their elongated shape, indicating that cell shapes did not depend on the expression of LH2 and or LH2 fusion proteins, which agreed well with the previous study (Fowler *et al*., 1995). However, the membrane morphologies were changed. Fig. 5a, 5b and 5c show the membrane morphology of *Rb. sphaeroides* CQU68, *Rb. sphaeroides* CQU68 harboring LH2 and *Rb. sphaeroides* CQU68 harboring LH2 with β-polypeptides-HNP3 fusion protein, respectively. The normal membrane morphology was lost in the *Rb. sphaeroides* CQU68, whereas, the normal membrane morphologies were restored in *Rb. sphaeroides* CQU68 harboring LH2 and *Rb. sphaeroides* CQU68 harboring HNP3 fusion protein, respectively. It has been suggested that the membrane morphology is probably affected by the presence of LH2 and PucC protein which plays very important roles in the formation of LH2. Restoration of the membrane morphology implies the expression of the HNP3 fusion protein.

Antimicrobial activity assay

After purification from *Rb. sphaeroides*, the antimicrobial activity of the HNP3 fusion protein was measured, as indicated in Fig. 6A, B and C, which represent Kan (50 mg/mL), HNP3 fusion protein and elution buffer used for the purification of HNP3 fusion protein, respectively. Clearly, the purified HNP3 fusion protein exhibited high antimicrobial activity against *E. coli*. On the other hand, the LH2 β-polypeptide only plays important roles in the formation of LH2 (Kiley & Kaplan, 1987). Consequently, the HNP3 peptide possessed high antimicrobial activity against *E. coli*, which agreed well with the previous study (Kagan, *et al*., 1994). Further antimicrobial activities tests against a wide range of microorganisms will be performed in the future.

In the present study, we harvested the HNP3 by SOE PCR and heterologously expressed the peptide in *Rb. sphaeroides* fused to LH2 β-polypeptide. To the best of our knowledge, this is the first time where *Rb. sphaeroides* was employed to express HNP3 and the purified HNP3 fusion protein exhibited antimicrobial activity, as suggested by the antimicrobial experiment against *E. coli*. The LH2 spectral properties at ~800 nm and ~850 nm could be used as an indicator in expression and purification for rapid detection of HNP3 fusion protein and thus the novel *Rb. sphaeroides* expression system exhibited advantages over other commercial expression system. In our next step we will focus on a large scale production of the HNP3 fusion protein and production of HNP3 of higher purity, as well as further testing of the fusion protein's antimicrobial activities against a wide range of microorganisms. Moreover, employing the novel *Rb. sphaeroides* expression system to express more human neutrophil peptides to further improve the expression system and trying to construct a novel expression system for heterologous expression of human neutrophil peptides will be in the center of our interests.

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