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## 嵌合鞭毛蛋白在大肠杆菌 BL21(DE3) 和沙门菌 SL5928 中的表达与组装分析

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胡茂志, 潘志明, 杨芸, 等. 嵌合鞭毛蛋白在大肠杆菌 BL21(DE3) 和沙门菌 SL5928 中的表达与组装分析. 生物工程学报, 2017, 33(8): 1335-1342.

Hu MZ, Pan ZM, Yang Y, et al. Expression and assembly of chimeric flagellins in *Escherichia coli* BL21(DE3) and *Salmonella* SL5928. Chin J Biotech, 2017, 33(8): 1335-1342.

**摘 要:** 鞭毛蛋白根据能否组装可以表达为单体或多聚体。目前, 关于它们之间的区别少有报道。文中, 将重组质粒 pET-fliC/M2e2 分别导入大肠杆菌 BL21(DE3) 和沙门菌 SL5928 中来表达嵌合鞭毛蛋白 mfliC/M 和 pfliC/M, 然后分析它们的组装特性。SDS-PAGE 结果表明, 两重组菌成功表达了嵌合鞭毛蛋白。透射电镜观察显示, 在重组大肠杆菌表面未见鞭毛, 而重组沙门菌表面呈现鞭毛。将嵌合蛋白纯化后, 圆二色谱 (Circular dichroism, CD) 分析表明, 两者的 CD 信号明显不同, pfliC/M 的 CD 信号与野生型鞭毛蛋白一致, 而 mfliC/M 基本上没有 CD 信号出现。动态光散射分析表明, mfliC/M 的聚集程度明显低于 pfliC/M。将嵌合蛋白转染小鼠腹腔细胞 3 h 后, 两者均能诱导产生 IL-1 $\beta$ , 但 mfliC/M 组高于 pfliC/M 组。以上结果对于鞭毛蛋白表达形式的选择具有重要的参考价值。

**关键词:** 鞭毛蛋白, 表达, 组装, 白细胞介素 1 $\beta$

**Received:** October 3, 2016; **Accepted:** March 9, 2017

**Supported by:** National Natural Science Foundation of China (Nos. 31320103907, 31372414), the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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国家自然科学基金 (Nos. 31320103907, 31372414), 江苏省优势学科资助。

网络出版时间: 2017-04-11

网络出版地址: <http://kns.cnki.net/kcms/detail/11.1998.Q.20170411.1707.003.html>

## Expression and assembly of chimeric flagellins in *Escherichia coli* BL21(DE3) and *Salmonella* SL5928

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**Abstract:** Flagellin can be expressed in monomeric or polymeric form based on assembly. The difference of these two forms of flagellin is less studied. In this experiment, recombinant plasmid pET-*fliC*/M2e2 was transferred into *Escherichia coli* BL21(DE3) and *Salmonella* SL5928 to express chimeric flagellin, *mfliC*/M and *pfliC*/M, respectively, and then their assembly characteristics were analyzed. Sodium dodecyl sulfate polyacrylamide gel electrophoresis results indicated that the two recombinant bacteria could successfully express chimeric flagellin. The transmission electronic microscope observation showed that no flagella were found on the surface of recombinant *E. coli*, whereas it was found for recombinant *Salmonella*. After purification, distinct circular dichroism spectra between them were found and *pfliC*/M showed the similar structure as wild-type flagellin, but not for *mfliC*/M. The dynamic light scattering assay also indicated that the polymerization of *mfliC*/M was much lower than that for *pfliC*/M. Three hours after transfection into mouse peritoneal macrophages, both could induce interleukin 1 $\beta$  secretion, but *mfliC*/M is stronger than *pfliC*/M. These data will be helpful for the selection of expression form of flagellin.

**Keywords:** flagellin, expression, assembly, interleukin 1 $\beta$

### Introduction

Bacterial flagellum is the motility device of bacterium and its elongated filament is composed primarily of many subunits of flagellin<sup>[1]</sup>. The N- and C-terminal regions of the *Salmonella typhimurium* phase 1 flagellin, encoded by the *fliC*<sup>i</sup> gene, have been reported to be highly conserved helix structure across different bacterial species to maintain the integrity of the filament structure<sup>[2]</sup>, whereas the middle regions are hypervariable regions (HVR). During bacterial flagellar filament assembly, flagellin has to be exported from the cytoplasm to the tip of the growing filament and undergoes a process from unfolding to folding state<sup>[3-4]</sup>. Because the folding and assembly process is essential for flagellin to be

expressed on the bacterial surface<sup>[5]</sup>, the recombinant flagellin can be acquired as unfolding form within the cytoplasm (monomer)<sup>[6-7]</sup> or folding form showing on the surface of bacteria (polymer)<sup>[5, 8-9]</sup>.

Bacterial flagellin is a potent trigger of host innate immune responses in eukaryotes<sup>[10-11]</sup>. Two forms of flagellin have been found to show different ascendancy in the immune response against exogenous antigen, although they have all been reported to show adjuvanticity<sup>[12-13]</sup>. Besides, the residues of flagellin which are involved in Toll-like receptor-5 signaling and NLRC4 (NLR family, CARD domain containing 4) inflammasome response are hidden in the flagellum and only monomer is accessible for recognition by host cells<sup>[14-17]</sup>. Hence the folding and assembly process of flagellin may

influence their biological function. It is reported that flagella combined with exogenous antigen can significantly promote the release of cytokine, when compared with flagella alone [12]. Thus, for the flagellin-specific response assay, the present of exogenous antigen is more practical for the vaccine development than flagellin alone.

Replacement of part of the HVR of flagellin by exogenous antigen is a potentially effective strategy for the vaccine development [18]. The monomeric flagellin expressed in *Escherichia coli* and the polymeric form directly isolated from *Salmonella* is usually used in the vaccine development [8,13,19-20]. Reports have shown that the linkage of flagellin with the ectodomain of matrix 2 protein of influenza A virus (M2e) is a good vaccine candidate [20-21]. In this experiment, in order to analyze the assembly and biological activities of two forms of flagellin, two tandem M2e genes were designed to replace the DNA sequence (607–684 bp) of the *fliC*<sup>i</sup> gene in HVR and the chimeric genes were expressed in *E. coli* and *Salmonella* to produce the monomer and polymer chimeric flagellin, respectively. Then, the protein conformation, polymerization and IL-1 $\beta$  release in the early stage of immunization were analyzed.

## 1 Materials and methods

### 1.1 Mice and bacteria

Ten six-week-old female C57BL/6 mice were obtained from the Comparative Medicine Center of Yangzhou University, China. This study was carried out in accordance with the regulations set forward by the Chinese Ministry of Science and Technology. The protocol was approved by the Committee on the Ethics of Animal Experiments of Yangzhou University (Permit number: 2007-0005). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. The recombinant plasmid pET-*fliC* containing *fliC*<sup>i</sup> gene of *S. typhimurium* LT2 in the *Bam*H I/*Xho* I sites of

pET30a+ was constructed by Dr. Hui Zhang [18]. *E. coli* BL21(DE3), *S. typhimurium* LB5000 and aflagellar *S. dublin* SL5928 were used in the following cloning procedures. The wild-type flagellin of *S. typhimurium* SL7207 was used as a control.

### 1.2 Construction of recombinant plasmids

The DNA fragment (5'-GATACTACGATTGCT TTAGACAATAGTACTTCCCTGCTGACCGAAGTT GAAACCCCGACCCGTAACGAATGGGAATGCCG TTGCTCCGATTCTCCGATGGCGGCGGCTGCTC CCTGCTGACCGAAGTTGAAACCCCGACCCGTA ACGAATGGGAATGCCGTTGCTCCGATTCTCCG ATAAATATTACGCCAAAGTTACCGTTACGGGG-3') was synthesized by GenScript Company (Nanjing, China). In this sequence, two M2e genes (4–72 bp) were connected with a DNA sequence, 5'-GGCGG CGGCTGC-3', to yield the M2e2 gene (underlined). At the two ends of the M2e2 gene, a partial HVR sequence of *fliC*<sup>i</sup> gene was included for overlap polymerase chain reaction (PCR) procedures. The 5'-terminal region of the *fliC*<sup>i</sup> gene, *fliC*-AC, was amplified from pET-*fliC* using the following primers: *fliC*-forward-A and *fliC*-reverse-C. The 3'-terminal region, *fliC*-DB, was amplified using the following primers: *fliC*-forward-D and *fliC*-reverse-B. The *fliC*-AC-M2e2 fragment was amplified using the following primers: *fliC*-forward-A and M2E2-reverse from *fliC*-AC and M2e2. The chimeric *fliC*/M2e2 gene was amplified using the following primers: *fliC*-forward-A and *fliC*-reverse-B from *fliC*-AC-M2e2 and *fliC*-DB (Table 1; Fig. 1). The PCR product was cloned into the *Bam*H I/*Xho* I sites of pET30a+ to yield the recombinant plasmid, pET-*fliC*/M2e2.

**Table 1 Primers used in this experiment**

Primer name	DNA sequence (5'–3')
<i>fliC</i> -forward-A	AGGGATCCATGGCACAAGTCATTAA
<i>fliC</i> -reverse-C	AGTACTATTGTCTAAAGCAATCG
<i>fliC</i> -forward-D	AAATATTACGCCAAAGTTACCG
<i>fliC</i> -reverse-B	TGCTCGAGTTAACGCAGTAAAGAGA
M2E2-reverse	CCCCGTAACGGTAACTTTGGCG

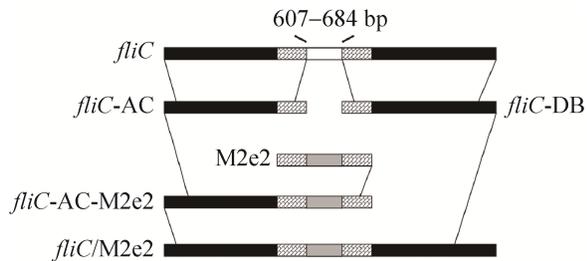


Fig. 1 Schematic construction of the chimeric gene *fliC/M2e2*.

### 1.3 Preparation of monomeric chimeric flagellin expressed in *E. coli* BL21(DE3)

The plasmid pET-*fliC/M2e2* was transformed into BL21(DE3). The recombinant bacteria, BL21(DE3)(pET-*fliC/M2e2*), were cultured in LB agar plates containing 50 µg/mL kanamycin overnight at 37 °C and the kanamycin-resistant colonies were then amplified by culturing with 1 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG). The recombinant bacterium BL21(DE3)(pET) was used as a negative control. Affinity purification of His-tagged monomeric chimeric flagellin (mfliC/M) was performed with a nickel bead column (Qiagen, Valencia, CA) according to the manufacturer's instructions after cell lysis by ultrasonication.

### 1.4 Preparation of polymeric chimeric flagellin expressed in *Salmonella* SL5928

The plasmid pET-*fliC/M2e2* was electroporated into LB5000 and then transferred to SL5928 by P22HTint transduction, as previously described<sup>[18]</sup>. Polymeric chimeric flagellin (pfliC/M) in the surface of kanamycin-resistant recombinant SL5928(*fliC/M2e2*) was extracted by acid treatment followed by ammonium sulfate precipitation, as previously described<sup>[13]</sup>.

### 1.5 Transmission electron microscopy

The growth of chimeric flagella in the surface of bacteria and purified proteins were observed by Tecnai-12 transmission electronic microscopy (Philips, Holland) after negative staining.

### 1.6 SDS-PAGE and Western blotting

The expression and purity of chimeric flagellin were confirmed by sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred onto nitrocellulose and treated with murine anti-flagellin polyclonal antibody for Western blotting assay.

### 1.7 Circular dichroism

To investigate the secondary structure of purified flagellin, pfliC/M and mfliC/M, the far-ultraviolet (UV) circular dichroism (CD) spectra were recorded using a J-810 spectropolarimeter (Jasco, Tokyo, Japan) using 0.2 cm path length cuvettes at 20 °C. The concentration of all samples used was 50 µg/mL in 10 mmol/L phosphate buffer saline (PBS), pH 7.4. The CD spectrum of PBS was used as baseline. The mean spectra of three repeated scans in the range between 190 nm and 250 nm, corrected by subtraction of the buffer signal, were obtained by taking points every 0.1 nm with 1 nm slit width.

### 1.8 Dynamic light scattering

To further investigate the polymerization of mfliC/M in solution, the dynamic light scattering (DLS) measurements for pfliC/M and mfliC/M were performed with an EEN 3690 Malvern Zetasizer (Malvern, Worcestershire, UK) at 20 °C. The concentration of all samples used was adjusted to 50 µg/mL in PBS (pH 7.4).

### 1.9 IL-1β release

Primary peritoneal exudate cells (PECs) of C57BL/6 mice were harvested by washing the peritoneal cavity with RPMI 1640 medium containing 10% fetal bovine serum (1640-FBS, Gibco, Carlsbad, CA), as previously described (Kumari and Saxena, 2011). Cells were plated in 96-well plates at  $2 \times 10^4$  cells/well and incubated for 3 h at 37 °C with 1 µg/mL *E. coli* lipopolysaccharide (LPS). The supernatants were removed and the cells were washed with 1640-FBS. pfliC/M and mfliC/M were then transfected respectively using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and incubated for 3 h (Simon and Samuel, 2008). The non-transfected cells were used as controls. The secretion of IL-1β in supernatant was detected using

a mouse enzyme-linked immunosorbent assay (ELISA) set (Biosciences, PharMingen, San Diego, CA).

### 1.10 Statistical analysis

Within each experiment, three to four replicate assays were conducted for each treatment. All statistical analyses were performed by Student's *t*-test using SPSS software (Version 13.0 for Windows, Chicago, IL). A value of  $P \leq 0.05$  was considered to be significant.

## 2 Results and discussion

### 2.1 Identification of two forms of chimeric flagellin

In the filament assembly of bacterial flagellum, the unfolding subunit protein flagellin has to be transported via a narrow channel, and then fold before being assembled into the growing filament<sup>[3-4]</sup>. The transport process is accompanied with the change of protein conformation. In this study, in order to express different forms of chimeric flagellin, the recombinant plasmid pET-*fliC/M2e2* containing the chimeric gene *fliC/M2e2* was designed to express as monomeric or polymeric flagellin using different expression systems, respectively.

For the expression of monomeric chimeric flagellin, the SDS-PAGE and Western blotting results indicated that m*fliC/M* was successfully expressed in recombinant bacteria BL21(DE3) (pET-*fliC/M2e2*) when compared to BL21(DE3)(pET) (Fig. 2A). No flagellum was found on the surface of recombinant *E. coli*. For the expression of polymeric chimeric flagellin, polymeric flagella were observed on the bacterial surface of SL5928(*fliC/M2e2*) when compared to SL5928 (Fig. 2B). The molecular mass of p*fliC/M* and m*fliC/M* were about 50 kDa and 60 kDa, respectively (Fig. 2C). After purified, the polymeric p*fliC/M* showed a filament shape similar to wild-type flagellin and was significantly different from monomeric m*fliC/M* by transmission electronic microscope (Fig. 2D).

In BL21(DE3), the 6×His-tag was fusion

expressed with *fliC/M2e2* for purification of monomeric m*fliC/M*. While in SL5928, the *fliC/M2e2* gene was integrated into the chromosome of SL5928 through homologous recombination<sup>[18]</sup>. In this study, the molecular weight between these two forms of chimeric flagellin was different. One reason may be due to the 6×His-tag in m*fliC/M*, besides post-translational modification.

### 2.2 Circular dichroism spectra and polymerization of two forms of chimeric flagellin

The folding and assembly of polymeric flagellin is one of the major difference compared with monomeric flagellin. CD spectroscopy is a common method for the analysis of protein structures in solution<sup>[22-23]</sup>. CD spectra between 190 and 250 nm are usually used to analyze the secondary structure of protein<sup>[23]</sup>. Moreover, the polymerization of proteins in solution can be determined by the size distribution by volume.

The far-UV CD measurements indicated that significantly different CD spectra between 190 and 250 nm were found between monomeric m*fliC/M* and polymeric p*fliC/M*. The CD signal of p*fliC/M* was stronger than that of m*fliC/M*. Similar CD spectra were observed between p*fliC/M* and wild-type polymeric flagellin of *S. typhimurium* SL7207 (Fig. 3A). The CD spectra of p*fliC/M* showed two negative peaks at 208 nm and 222 nm. This indicated that the helix conformation is its major secondary structure.

The polymerization of proteins in solution measured by DLS indicated that the mean dynamic radius of p*fliC/M* was larger than that of m*fliC/M*, though a few polymers were present in the m*fliC/M* solution (Fig. 3B). This indicated that the polymerization degree of p*fliC/M* was higher than that of m*fliC/M*.

The significant difference in transmission electronic microscope observation, CD spectra and polymerization between m*fliC/M* and p*fliC/M* may be due to the process of assembly. The terminal regions of flagellin are conserved helix structure to maintain the integrity of the filament structure<sup>[2]</sup>.

The secondary structure of pfliC/M in this study showed typical helix conformation confirmed this point. Whereas only low-level polymerization of mfliC/M were found *in vitro*, thus we suppose that the polymerization of pfliC/M *in vivo* is a complex process. The middle region of flagellin is a HVR

varying greatly in size and composition in different strains, and the insertion of exogenous antigen into HVR does not interfere with the assembly and export function<sup>[1,3-4]</sup>. This study also verified that replacement of part of the HVR by M2e did not interfere with the conformation of polymeric flagellin.

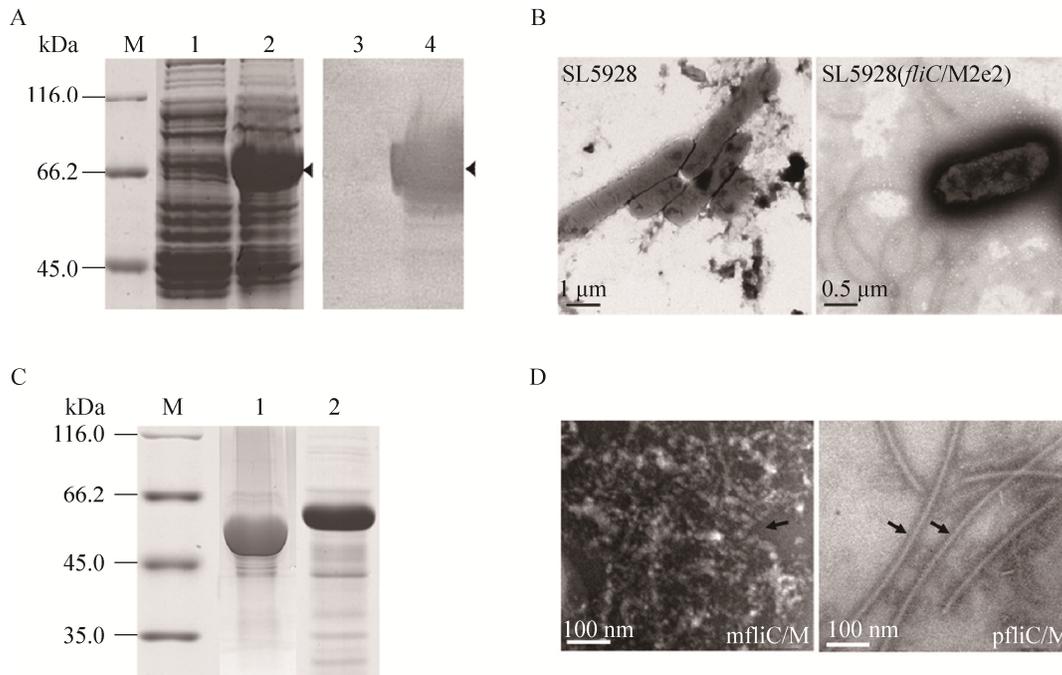


Fig. 2 Generation of chimeric flagellin pfliC/M (polymer) and mfliC/M (monomer). (A) SDS-PAGE (lane 1, 2) and Western blotting (lane 3, 4) analysis of recombinant bacteria induced with IPTG. M: standard molecular weight markers; lane 1 and 3: BL21(DE3)(pET); lane 2 and 4: BL21(DE3)(pET-*fliC/M2e2*). (B) Transmission electron microscopy observation of SL5928 and SL5928(*fliC/M2e2*) after negative staining. (C) SDS-PAGE of purified proteins. M: standard molecular weight markers; lane 1: pfliC/M; lane 2: mfliC/M. (D) Transmission electron microscopy observation of purified mfliC/M and pfliC/M after negative staining.

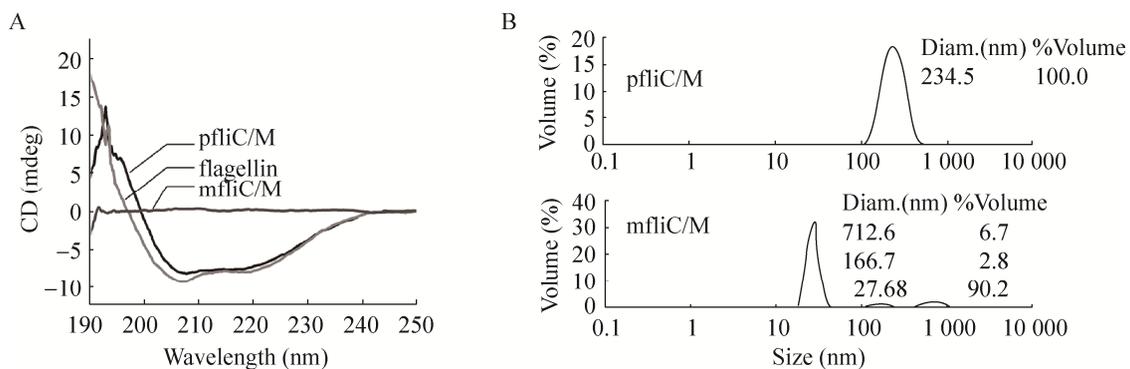


Fig. 3 Circular dichroism (A) and dynamic light scattering (B) assay of pfliC/M and mfliC/M. Results represent one of four independent experiments.

### 2.3 IL-1 $\beta$ release of PECs after transfection

LPS does not activate NLRC4 [24], but can induce the production of pro-IL-1 $\beta$  [25]. The C-terminal amino acid sequence of flagellin, which was hidden in the inner of filament, can be sensed by NLRC4 in macrophages and then trigger the maturation and release of IL-1 $\beta$  [4,17]. Thus, the level of IL-1 $\beta$  secreted by macrophages can be used to indicate the activation of the NLRC4 signaling pathway. Report has shown that the secretion of IL-1 $\beta$  can be stimulated after transfection of monomeric flagellin to PECs [26-27]. In the study, three hours after transfection, the IL-1 $\beta$  assay indicated that pfliC/M and mfliC/M could significantly induce the secretion of IL-1 $\beta$  in PECs compared with the untransfected group ( $P < 0.05$ ). The secretion level of IL-1 $\beta$  triggered by pfliC/M was lower than that for mfliC/M ( $P < 0.05$ ) (Fig. 4). The relatively higher level of IL-1 $\beta$  secretion induced by mfliC/M may be due to the direct recognition by NLRC4 in the early stage, whereas polymeric flagellin must dissociate to release the monomeric form of flagellin first. While the detailed mechanism need to be further studied.

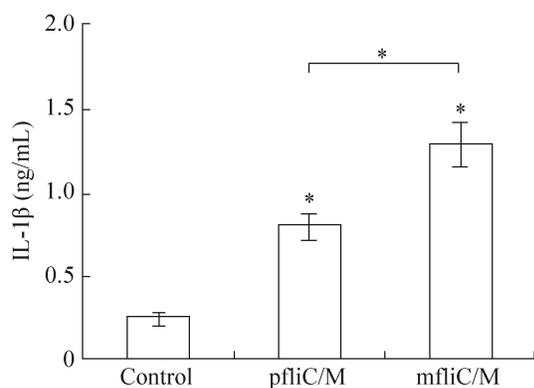


Fig. 4 IL-1 $\beta$  secretion. Primary peritoneal exudate cells ( $2 \times 10^4$ /well) from C57BL/6 mice were incubated with *E. coli* LPS (1  $\mu$ g/mL) for 3 h to induce the production of pro-IL-1 $\beta$ . Then, pfliC/M or mfliC/M was transfected using Lipofectamine 2 000 and incubated for 3 h. The concentration of IL-1 $\beta$  in supernatant was detected by ELISA. Non-transfected cells were used as control. These experiments were repeated three times.

### 3 Conclusion

Flagellin can be recognized by the innate immune system and has been used as adjuvant in the vaccine development [28]. The different expression and assembly assay of chimeric flagellin found in this study will be beneficial for their use in the future.

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(本文责编 郝丽芳)