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Molecular Cloning and Expression of a Phase I Flagellin Gene from *Salmonella enterica* serovar Choleraesuis

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ABSTRACT: P56, a protein with a molecular weight of 56 kDa, was isolated from the cell-free supernatant of *Salmonella enterica* serovar Choleraesuis CH12440 (*Salmonella* Choleraesuis CH12440) culture. Comparison of the N-terminal amino acid sequence of P56 with the sequences in GenBank indicated that it was flagellin, the product of *fliC* gene and the subunit of flagellar filament. The gene encoding P56 was cloned and expressed. Although the gene displayed a 99.2 % nucleotide sequence homology and a 98.6 % amino acid sequence homology with other *S.* Choleraesuis phase-1-c flagellin genes, there was an insert of 36 nucleotides absent in other phase-1-c flagellin genes. The expressed P56 protein was toxic to macrophage cell Raw264.7, and caused the programmed death of the cells *in vitro*.

KEY WORDS: Cytotoxin, Flagellin, *Salmonella enterica* serovar Choleraesuis, Programmed death.

INTRODUCTION

Salmonella enterica serovar Typhi (*S.* Typhi), *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *Salmonella enterica* serovar Choleraesuis (*S.* Choleraesuis) are three major pathogenic *Salmonella* species. They are etiological agents of typhoid fever, diarrhea and abdominal pain, and septicemia, enteritis and pneumonia, respectively (Nnalue, 1990; Salyers and Whitt, 1994; Chang and Tsai, 1996). *S.* Choleraesuis normally is a swine pathogen and occasionally causes systemic infection in human (Chang and Tsai, 1996). The endotoxin is considered a major virulence factor of *Salmonella* spp. and is the cause of death in people with systemic infection. Enterotoxin has also been reported for a large number of *Salmonella* serotypes and, at least in part, responsible for the loss of electrolytes and fluid from small intestine (Giannella et al., 1975; Caprioli et al., 1982; Singh et al., 1985).

In the past decade, the cytotoxins produced by enteric pathogens have been increasingly investigated. These toxins have been referred to as verotoxins because they are cytotoxic to Vero cells (Konowalchuk et al., 1977). Some cytotoxins are lethal to mammalian cells, such as Shiga toxin produced mainly by *Shigella dysenteriae* serotype 1 (Bartlett et al., 1986) and the closely related Shiga-like toxin (SLT) produced by *Escherichia coli* (Cleary et al., 1985; Marques et al., 1986). A similar cytotoxin is produced by *Campylobacter jejuni* (Guerrant et al., 1987). Although evidence has evolved regarding the role of cytotoxins produced by toxigenic organisms in the pathogenesis of particular diseases, very limited data are available concerning the cytotoxin produced by *Salmonella* spp. We previously isolated a cytotoxin from the cell-free supernatant of *S.* Choleraesuis culture, named P56. It caused degeneration and necrosis of mouse macrophage cell line P388-D1 *in vitro*, and hepatomegaly, splenomegaly and pneumonia in ICR mice following intravenous injection (Chang and Tsai, 1996). In this study, we demonstrated that cytotoxin P56 was flagellin, the subunit of flagellar filament.

Flagella are thin, hair-like and rigid appendages of bacteria, and are bacterial locomotive structures. A flagellum has three basic parts: The outmost and longest part is a filament which consists of around 20,000 protein subunits of a single protein called flagellin (FliC) with a molecular weight of 50 to 60 kDa. The flagellin is made within the cell and then passed along the hollow core of the helical filament to be added to the distal end. The filament is attached to a slightly wider and shorter hook which arises from the basal body. The basal body anchors the flagellum to the cell wall and plasma membrane, and is the motor of the flagellum (Kondoh and Hotani, 1974; Macnab, 1996).

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Flagella have been recognized as one of the potential virulence factors of microorganisms. However, most suspicion has been focused on motility necessary for colonization and the aid of virulence factor secretions (Penn and Luke, 1992; Josenhans and Suerbaum, 2002; Hirano et al., 2003). In this communication, we reported the cloning and characterization of the gene encoding P56. The expressed P56 was directly involved in the pathogenesis of bacteria.

MATERIALS AND METHODS

Bacterial strain

Salmonella Choleraesuis CH12440 was isolated from lungs, hilus lymph node of lungs, spleen, liver, and gallbladder of pigs naturally infected with systemic septicemic salmonellosis (Chang and Tsai, 1996).

Cell line

Murine macrophage cell line RAW264.7 was purchased from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium supplemented with 10 % fetal calf serum.

Amino acid determination

The purified P56 protein showed a single band on a 9% SDS-polyacrylamide gel (SDS-PAGE) was used for amino acid sequence determination. The N-terminal amino acid sequence of protein was determined by Edman degradation using an ABI Procise model 491 protein sequencer (Applied Biosystem, USA) according to the method described by Tam et al. (1998).

Bacterial genomic and plasmid DNA isolation

Genomic DNA was isolated using the procedure described by Roussel and Chabbert (1978) with some modifications. A 300 mL overnight culture in LB broth (Luria-Bertani broth: 10g bacto-tryptone, 5g bacto-yeast extract, 10 g NaCl per liter) was harvested at 4,000*xg*. Pellet was suspended in 4 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), then 100 μL lysozyme (10 mg/mL in TE) and 100 μL RNAase A (10 mg/mL in water) were added. The solution was incubated at 37℃ for 30 min. Incubation was continued for 1 hr after addition of 1.5 mL proteinase k/sarkosine (5 mg proteinase k and 0.1 g sarkosine/mL TE). After centrifugation at 25,000*xg*, 20 min, 4 ℃, the supernatant was transferred to a new tube and extracted with phenol/chloroform/isoamlyalcohol (25:24:1) twice, and then chloroform/isoamylalcohol (24:1) two times. The aqueous layer was transferred to a new tube and mixed with 0.04 volumes of 5 M NaCl and 2.5 volume of 100% alcohol. The DNA was precipitated at 12,000*xg*, 4℃, 20 min after chilling at –20℃ for 1 hr. The pellet was rinsed with 75% alcohol, air dried and then resuspended in 1.5 mL TE.

Plasmid DNA was prepared by alkaline extraction procedure (Sambrook et al., 1989). For sequencing, plasmid DNA was isolated by QIAprep Spin Miniprep Kit (QIAGEN, Germany).

DNA probe preparation

After P56 was identified by partial protein sequencing as flagellin, two oligonucleotides (SC1: 5'-TGACCCAGAATAACCTG-3' and SC2: 5'-GAGTCGAGGTCAGACTG-3') were made according to the conserved regions of phase-1 and phase-2 flagellin genes of several *S.* Choleraesuis and *S.* Typhimurium strains (Joys, 1985; Vanegas and Joys, 1995; Wei and Joys, 1985). A 295-bp DNA fragment, named fragment FL295 was amplified on the *S.* Choleraesuis CH12440 genomic DNA using this pair of oligonucleotides in a PCR reaction. The PCR reaction was carried out as described previously (Ho et al., 1992). The fragment was cloned into pUC18 at *Sma*I site and sequenced to confirm the existence of the *fliC* sequence.

Genomic library construction and screening

Library construction and screening were done as described previously (Ho and Chang, 2000; Ho et al., 2001). The *S.* Choleraesuis CH12440 DNA was partially digested with *Eco*RI and cloned into λzap (ZAP Express System, Stratagene). The library contained 1.1×10^5 plaque-forming unit (pfu) with greater than 95% recombinants determined in the presence of isopropyl-*β*-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chioro-3-indolyl-*β*-D-galactoside (Xgal). The library was screened for the *fliC* gene by plaque hybridization using ^{32}P-labeled fragment FL295 as probe. The positive plaque areas were selected and re-screened until a single, isolated plaque could be picked up. A clone named λSC170 was obtained. The recombinant λ DNA was then converted into the phagemid pSC170.

Construction of flagellin gene in expression vector

In order to express P56 protein, a PCR DNA fragment containing the coding region of *fliC* was amplified on pSC170 DNA using oligonucleotides of Sal-B and Sal-MS (Sal-B: 5'-GCGGGATCCATGGCA CAAGTAATCAACAC-3' and Sal-SM: 5'-TCC CCCGGGTTAACGCAGTAAAGAGAGGAC-3'. The underlined sequences were the recognition site for

*Bam*HI and *Sma*I, respectively.), and cloned into expression vector pGEX-6P-1 (Amersham Biosciences Ltd) to generate a recombinant plasmid pSC15. The cloned DNA of pSC15 was subjected to sequencing to insure its entirety. The recombinant plasmid was used to transform *E. coli* DH5α.

Expression of the recombinant protein

The recombinant protein was expressed as follows: 2.5 mL of an overnight-culture were added to a 47.5 mL LB broth containing 50 μg/mL of ampicillin. The bacteria grew at 37℃ until the OD_{600} of the culture was 0.7 to 0.9, then an IPTG solution was added to a final concentration of 1 mM. The cultivation was continued for 2 hr. The bacteria were harvested at 10,000*xg*, 4℃, 10 min and resuspended in 1 mL cold lysis buffer (50 mM NaH_2PO_4/300 mM NaCl, pH 8.0) containing 1 mg lysozyme, and then incubated on ice for 30 min. The cells were sonicated on ice for 15 sec each with cooling pause of 15 sec in between at 30 W (Ultrasonic processor, grade 5, Heat systems, NY) until the lysate became transparent and yellowish. The bacterial debris was removed at 10,000x*g*, 4℃, 30 min. The recombinant proteins were further purified by Glutathione Sepharose 4B column according the instruction of manufacture (Amersham Biosciences Ltd) and/or then cleaved by PreScission protease. Proteins were analyzed on a 12% SDS-PAGE. Proteins were stored at –70℃ if they were not used immediately.

Purification of cytotoxin, preparation of antibodies, Western blot analysis and DNA sequence determination and cytotoxicity test

These experiments were performed as described previously (Ho et al., 2003).

Nucleotide sequence Accession numbers

The nucleotide sequence of *fliC* was deposited in GenBank (accession no. AF159459).

RESULTS

Cloning the gene encoding protein P56

In order to clone the gene coding for P56, the purified protein was subjected to the amino acid analysis by Edman degradation using an ABI Procise model 491 protein sequencer. A sequence of AlaGlnValIleAsnThrAsnSerLeuSerLeuLeuThrGln Asn from the N-terminus was obtained and demonstrated to be a part of amino acid sequence of flagellin, the product of *fliC* gene and the protein subunit of flagellar filament by comparison with the proteins deposited in GenBank.

A DNA fragment, named fragment FL295, containing 295 nucleotides of *fliC* was amplified in a PCR reaction on *S.* Choleraesuis CH12440 genomic DNA using a pair of primers (SC1 and SC2) designed according to the conserved regions of bacterial *fliC* genes. The fragment FL295 was used as a probe to screen a λ_{ZAP} *S.* Choleraesuis genomic library, and a positive clone (λSC170) containing a 3,787-bp insert DNA was obtained.

Comparison of the nucleotide sequence of the insert DNA with sequences of the genes in GenBank showed that it had a high homology with the sequences of *fliD, fliC, fliU* and *fliV* genes of *Salmonella enterica* serovar Muenchen (Doll and Frankel, 1993; Ho and Chang, 2000).

Characteristics of *fliC* gene

The *fliC* gene contained 1,506 nucleotides encoding a 501-amino acid flagellin protein (FliC) with a predicted molecular weight of 52 kDa and a pI of 4.7. The gene had an ATG as a start codon which was preceded by AGGA Shine-Dalgarno sequence, and flagellar operon-specific –10 (GCCGATAC) and –35 (TAAA) promoter consensus sequences (Kutsukake et al., 1990). Comparison with the *S.* Choleraesuis phase-1-c flagellin genes published by Wei and Joys (1985), the genes had a 99.2 % similarity in nucleotide sequence and 98.6 % in amino acid sequence. The high homology indicated our *fliC* was a phase-1 flagellin gene. However, there was a region of 36 nucleotides absent in other *S.* Choleraesuis phase-1-c flagellin genes (Fig. 1).

Expression of the recombinant proteins

E. coli DH5α was transformed with plasmid pSC15 to over-express the recombinant proteins in the presence of IPTG. The cell lysate was analyzed on a 12% SDS-PAGE. Figure 2 showed that a protein with a molecular weight of approximate 56 kDa (named as P56A, lane 4) was detected in the lysate of pSC15-transformed cells, but not in that of pGEX-6P-1 transformed ones. Western blot analysis shown in Fig. 3 indicated that expressed protein P56A could be stained by the antibody raised against purified P56.

Cytotoxicity assay for the P56 and P56A proteins

The cytotoxicity of P56 and P56A were tested on mouse macrophage cell line Raw264.7 by the MTT method. The macrophage cells were cultured in RPMI 1640 medium supplemented with 10 % fetal calf serum at 37℃ for 24 hr, and then different amounts of P56 or P56A were added. Figure 4 showed that the survival rate decreased significantly in cells treated with P56 and P56A.

Fig. 1. The nucleotide sequence of flagellar genes in pSC170. Complete nucleotide and deduced amino acid sequences of *fliC* were shown. ORFs of *fliD* and *fliU* were displayed by broken lines. Numbering of the nucleotide sequence commenced with the *Eco*RI site of insert DNA. Putative –10 and –35 regions of promoters, and Shine-Dalgarno (SD) sequences of each gene were underlined and indicated. The initiation and termination codons were boxed. The sequence of 36 nucleotides in *fliC*, absent from the same genes of other *Salmonella* spp. was underlined (nucleotide 1681 to 1716). The nucleotides of *fliC* in pSC15 different from those in pSC170 were $C^{806}\rightarrow A$, $T^{809}\rightarrow C$, $T^{812}\rightarrow C$, and $A^{1089}\rightarrow T$.

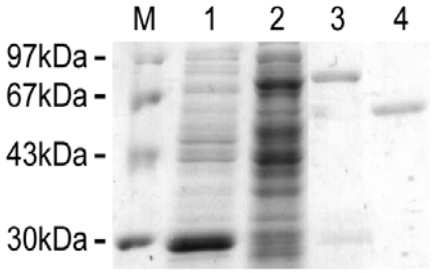

Fig. 2. Expression of FliC from pSC15 in *E. coli* DH5α. Proteins were extracted from cells by a buffer containing SDS, separated on a 12% SDS-PAGE and then stained with Coomassie blue. Lane M, protein size markers (kDa). Lane 1, proteins (50 μg) from pGEX-6P-1 transformants. Lane 2, proteins (50 μg) from pSC15 transformants. Lane 3, fusion protein of GST and P56A purified from pSC15 transformants by a glutathione sepharose 4B column, (20 μg). Lane 4, P56A, purified fusion protein of GST and P56A cleaved by PreScission protease (20 μg). Expression of proteins was induced by 1mM IPTG for 2 hr.

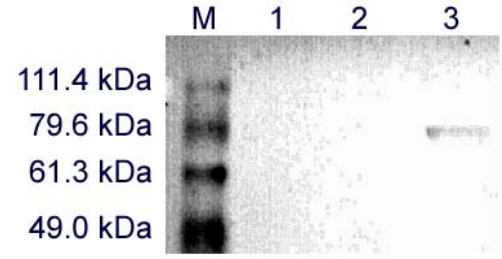

Fig. 3. Western blot analysis of expressed proteins. Lane M, protein size markers (kDa). Lane 1, cell lysate of *E. coli* DH5α. Lane 2, cell lysate of *E. coli* DH5α harboring pGEX-6P-1. Lane 3, cell lysate of *E. coli* harboring pSC15. Expression of proteins was induced by 1mM IPTG for 2 hr. The antibody was prepared against purified P56 from culture supernatant of *S.* Choleraesuis.

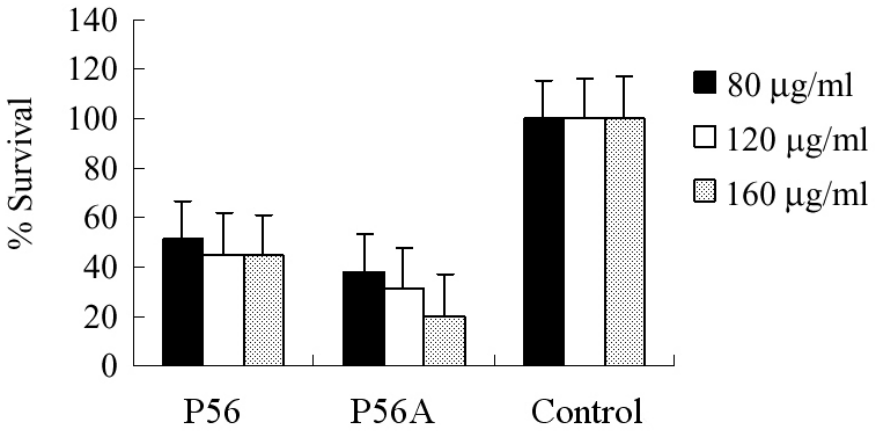

Fig. 4. Cytotoxic effect of P56 and P56A on mouse macrophage Raw264.7 cell line. The Raw264.7 cells were cultured in RPMI 1640 medium containing different amounts of P56 and P56A. The effect of protein on the cell viability was determined by the MTT method. P56 protein was isolated from culture supernatant. P56A was isolated from the purified fusion proteins (GST-P56A) cleaved by PreScission protease. The experiments were performed three times in triplicate.

The fusion protein of P56A and glutathione S-transferase (GST) had the same toxicity of P56A (data not shown).

To further characterize the mechanism of cell death resulting from P56, the DNA isolated from P56-treated and untreated Raw 264.7 cells were electrophoresed on a 1% agarose gel. DNA fragmentation in the ladder pattern was noted in P56-treated cells but not clearly identified in untreated ones as shown in Fig. 5. The result suggested that the cell death caused by P56 was involved apoptosis.

DISCUSSION

The production of toxin is one of major virulence factors involved in the pathogenesis of *Salmonella* spp. The enterotoxins are reported to be associated with some *S.* Typhimurium strains and the symptoms of typhoid fever probably result from the endotoxin, lipopolysaccharide (LPS) of the outer membrane-mediated release of cytokines. However, considerable tissue destruction observed during *Salmonella* infection cannot be explained by damage due to endotoxin or enterotoxin (Salyers and Whitt, 1994; O'Brien and Holmes, 1996). In fact, *Salmonella* cytotoxins have long been suspected. In 1983, Koo and Peterson reported a heat-labile cytotoxin that inhibited protein synthesis in cell-free extract of *Salmonella*. A membrane-bound cytotoxin of *S.* Typhimurium was reported by Reitmeyer et al. (1986). The production of cytotoxins could account for the tissue damage (Reitmeyer et al., 1986). Libby et al. (1990) cloned a gene encoding cytotoxin with haemolysin activity from *S.* Typhimurium. Kita et al. (1993) reported a cytotoxic protein of 32 kDa isolated from L-form *S.* Typhimurium, with an isoelectric point (pI) of 6.4. This toxin was thermolabile and sensitive to trypsin. It could stimulate macrophages to produce a significant amount of tumor necrosis factor alpha (TNF-alpha) and had a cytolytic effect at concentrations higher than 0.7 μg/mL. Rumeu et al. (1997) characterized another outer membrane-bound, heat-stable cytotoxin produced by *S. enterica* serovar Eenteritidis (*S.* Eenteritidis) and *S.* Typhimurium, that inhibited protein synthesis in culture cells as well as in the intestinal mucosa.

Fig. 5. DNA fragmentation of Raw 264.7 cells. DNA was isolated from Raw 264.7 cells treated (lane 1) or untreated (lane 2) with P56 at a concentration of 80 μg/mL for 72 hr, and then electrophoresed on a 1% agarose gel.

Previously, we have isolated a cytotoxin, P56, with a molecular weight of 56 kDa and a pI of 6.2 from the cell-free supernatant of the *S.* Choleraesuis CH12440 culture. This cytotoxin caused hepatomegaly, splenomegaly and interstitial pneumonia in mice. It also caused degeneration and necrosis of mouse macrophage cell line P388-D1 *in vitro*. A negative Shwartzman's reaction indicated that it was not an LPS endotoxin (Chang and Tsai, 1996). P56 can be detected as early as 3 hr in the supernatant of the culture. The secretion of this protein increased by a factor of 3.2 when the cells were grown in BHI supplemented with 10 mM $CaCl_2$, compared to that in the medium without $CaCl_2$ (Ho et al., 2003).

In this communication, the gene encoding P56 was cloned and characterized. The partial amino acid sequence of P56 indicated that it was the protein flagellin of *S*. Choleraesuis CH12440. A fluorescence confocal microphotograph showed that the periplasm and flagella of *S.* Choleraesuis were stained with the antibody raised against P56 (data not shown). This result agreed with that flagellin is secreted into the periplasm where it is exported to assemble the flagellum.

Flagella are reported not only necessary for motility but also important for the virulence and invasion of *Vibrio cholerae* (Freter and Jones, 1976; Attridge and Rowley, 1983) and *Vibrio vulnificus* (Lee et al., 2004). Drake and Montie (1988) demonstrated that Fla^- variants of *Pseudomonas aeruginosa* had a diminished virulence and immotile Fla^+ variants lost virulence in animal models. In *C. jejuni*, the flagella played an important role in attachment and invasion (Wassenaar et al., 1991). It was reported that two flagellar genes, *fliA* and *flgM* involved in regulation of the flagellar operon might be involved in regulating the expression of virulence genes in *S.* Ttyphimurium (Carsiotis et al., 1989; Gillen and Hughes, 1991; Schmitt et al., 1994) and that flagella could help *S.* Typhimurium to survive within murine macrophages (Weinstein et al., 1984). Analysis of nonflagellated *Salmonella* strains revealed a correlation between the ability to induce TNF-alpha and the expression of the phase-1 filament subunit protein FliC. Furthermore, *Salmonella* FliC could restore the TNF-alpha inducing phenotype in *E. coli*, which otherwise lacked the activity (Ciacci-Woolwine et al., 1998). Flagellar formation was also suggested to contribute to the virulence of *Erwinia carotovora* subsp. *carotovora* significantly in plants (Matsumoto et al., 2003).

The overlap between motility and the secretion of virulence proteins has been noted in many organisms. There are significant homologies between several flagellar proteins and proteins involved in the expression of virulence genes, the export of virulence proteins, or both, in plant and animal pathogens. The type III secretion mechanism of bacteria exports many virulence effector proteins involved in subversion of eukaryotic cells. The system requires more than 20 proteins, and its core components are homologous to proteins essential for the assembly of flagella (Harshey and Toguchi, 1996; Bennett and Hughes, 2000).

Although motility and flagella were suggested to play role in bacterial virulence, the role of the flagellum in pathogenesis has not yet been proved directly. Our data provide the evidence that flagellum is directly involved in the pathogenesis of bacteria.

Note: The whole genome of a local *S.* Choleraesuis isolate with the insert of 36 nucleotides in *fliC* was sequenced and published after our work was completed (Chiu et al., 2005).

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豬霍亂沙門氏桿菌第一型鞭毛素基因的選殖與表現

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摘　　要

P56 是從豬霍亂沙門氏桿菌 CH12440 (*Salmonella* Choleraesuis CH12440) 培養基上清液純化出來，分子量為 56 kDa 的蛋白質。將 P56 的 N 端胺基酸定序並與基因資料庫(GenBank)比對，發現它是一種鞭毛素(flagellin)。此蛋白為 *fliC* 基因的產物，是構成鞭毛絲狀體的次單元。我們將 P56 蛋白的基因選殖於大腸桿菌並表現其蛋白。雖然這個基因與其他 *S.* Choleraesuis 第一型(phase-1-c)鞭毛素基因之核苷酸序列有 99.2%的相似性，胺基酸序列有 98.6%的相似性，但是在 P56 基因中有一連續 36 個核苷酸是其他 *S.* Choleraesuis 第一型鞭毛素基因所沒有。這個選殖基因表現出來的蛋白質對小鼠巨噬細胞 Raw264.7 具有毒性並會造成細胞的凋亡。

關鍵詞：細胞毒素、鞭毛素、豬霍亂沙門氏桿菌、細胞凋亡。

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