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Biological function of *hpsh4590* localized in the plasticity zone of *Helicobacter pylori*

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ABSTRACT

The aim of this study was to determine the biological function of *hpsh4590* in *Helicobacter pylori*. After Hpsh4590 was expressed using a prokaryotic expression system, the cytotoxic effects and IL-8 production of Hpsh4590 were analyzed by co-culturing with GES-1 cells. Meanwhile, the antibody of rHpsh4590, produced by immunizing rabbit, was used for localization and protein interaction studies. Hpsh4590 fusion protein was expressed successfully in *Escherichia coli* Rosetta (DE3), and the polyclonal antibody was produced at high titers. The MTT assay showed that the inhibition ratio of GES-1 cells cultured with 0.1 μg/mL rHpsh4590 (3.02% ± 0.02%) was significantly lower than that of 20 μg/mL rHpsh4590 (57.57% ± 0.03%, $p < 0.01$), while DAPI staining showed the cytotoxic effects of rHpsh4590 for GES-1 cells. The up-regulation of cleaved caspase-3 and cleaved PARP was observed after GES-1 cells co-cultured with rHpsh4590 by Western blot. Co-culturing of GES-1 cells with rHpsh0459 (20 μg/mL) led to significant production of IL-8 at 12 h(1097.74 ± 212.37 pg/mL) and 24 h (1379.55 ± 209.58 pg/mL) then at 6 h(134.68 ± 14.64 pg/mL, $p < 0.01$). These observations suggest that the cytotoxicity of Hpsh4590 occurred in a concentration dependent manner, which is related with IL-8 secretion from gastric mucosal epithelial cells. Hpsh4590 was found localized in the membrane and the periplasm of *H. pylori*, interacted with zinc finger protein and methionine ABC transporter ATP-binding protein, and potentially regulates DNA uptake or transfer.

1. Introduction

Helicobacter pylori (*H. pylori*) is a cause of many common chronic infections in humans and is associated with the development of gastric cancers [1,2]. Currently, several virulence factors including urease, vacuolating cytotoxin (*vacA*) and cytotoxin-associated gene A (*cagA*) have been well-characterized for their roles in bacterial colonization and gastric inflammation during *H. pylori* infections [3]. Noticeably, highly virulent *H. pylori* strains encode *cag* pathogenicity island (*cag*PAI), which leads to the expression of a type IV secretion system (T4SS) to form a syringe-like pilus structure and injection of virulence factors such as CagA into host target cells.

The T4SS of *Agrobacterium tumefaciens* is a prototype system that consists of 11 *virB* genes (*virB1~virB11*) and *virD4*, which are assembled into the transport apparatus and is necessary for the delivery of proteins or nucleoprotein complexes into target cells. For *H. pylori*, the most studied T4SS are the *cag*PAI system containing *virB4*, *virB7~B11* and *virD4*, and the ComB system as the DNA import system that contains the homologues of all *vir* genes, except *virB1* and *virB5* [4,5]. Functionally, *cag*PAI is involved in the induction of proinflammatory cytokine IL-8 and activation of transcription factors such as NF-κB in gastric epithelial cells [6].

Duodenal ulcer promoting gene A (*dupA*) was first described in 2005 and was found localized in the plasticity zone of *H. pylori* and predicted to form a new T4SS with other *vir* genes [7,8]. The putative T4SS named as *tfs3* consists of *virB*/*D* homologues, and some evidences support *tfs3* to play a role in general bacterial conjugation and transformability [9]. This type of T4SS has been previously

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observed in three complete *H. pylori* strains, which includes Shi470, Cuz20, and G27 [10].

The *hpsh4590* gene in the plasticity zone of *H. pylori* is high homologous with the *virB10* gene, while six *vir* genes around *hpsh4590* are homologues with *virB4*, *virB8*, *virB9*, *virB11*, *virD4*, *and virD2* [11]. It has been proposed that these *vir* genes might form *tfs3* that plays a role in DNA export, bacterial conjugation, and protein secretion [12]. Hpsh4590 is also a VirB10 homologue of CagY. CagY is VirB10 homologue in *H. pylori*, which is associated with pilus structures between *H. pylori* and host cells [13]. Few studies had elucidated the function of *hpsh4590*. Thus, in this study, we further characterized *hpsh4590* using immunological and molecular biology techniques.

2. Materials and methods

2.1. Bacterial strains and growth conditions

H. pylori strain WH21 with intact *tfs3* was cultured in our laboratory at 37 °C under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) on Karmali campylobacter agar base plates containing 8% sheep blood. *Escherichia coli* DH5α and Rosetta (DE3) strains were grown in Luria–Bertani broth (LB) or LB agar medium at 37 °C.

2.2. Bioinformatics analysis

The physical and chemical properties of Hpsh0459 were predicted by using ProtParam (*http://web.expasy.org/protparam/*). Signal peptide and antigen epitope were predicted by SignalP 4.1 server (*http://genome.cbs.dtu.dk/services/SignalP/*) and BepiPred 1.0 Server *(http://www.cbs.dtu.dk/services/BepiPred/)*, respectively. The functional domain of Hpsh4590 was analyzed by NCBI CDD, and the advanced structure was predicted by Phyre2.0 [14,15].

2.3. Expression, Purification, and antibody production

The sequence of *hpsh4590* from *H. pylori* strain WH21 was amplified using primers F (5′-GGATCCAGTGAAAACAAACTTTTGAGAAC-3′) and R (5′- CTCGAGGACTTCATTGTTTTTAGGGATAG -3′). The amplified targeted DNA of *hpsh4590* was cloned into the PMD®18-T vector (Takara, Japan), and sequenced by Life Technologies Corporation. After generating the recombinant expression vector, pET28a (+)-*hpsh4590* was transformed into *E. coli* Rosetta (DE3) and expression of rHpsh4590 was induced by IPTG and purified using Ni-nitrilotriacetic acid resin [16]. The LPS was removed by Detoxi-Gel™ Endotoxin Removing Gel (Thermo Scientific), and the purified proteins were assayed for LPS by Pyrotell™ [17]. The polyclonal antibody of rHpsh4590 was produced by immunizing rabbit [18].

2.4. Bacterial subcellular localization

Analysis of bacterial subcellular localization was performed as previously described [19]. *H. pylori* strain WH21 was grown on Karmali campylobacter agar plates under microaerobic conditions for 72 h and collected with brucella broth medium supplemented with 5% fetal bovine serum (FBS). The bacteria were resuspended in cold 10 mM Tris–HCl buffer containing sucrose (0.75M), Lysozyme (100 μg/L) and PMSF (0.1 mM). Subsequently, the suspension was gently and slowly diluted with 2 volumes of cold EDTA (1.5 mM) in 10min. After centrifuging at 5,000 g for 10min in 4 °C, the supernatant was periplasm. The lysate (cytosolic and membrane components) was further ultracentrifuged (100,000 g, 1 h, 4 °C) after ultrasonic processing. The cytoplasmic and membrane components were separated respectively. Each fraction was analyzed by immunoblot analysis using anti-Hpsh4590 antibody (1:2000).

2.5. Cell proliferation assay and determination of IL-8 secretion

GES-1 cells were cultured in 96-well plates (1×10^5 cells/well) for 14 h and treated with purified rHpsh4590 (0.1, 1.0, 2.5, 5.0, 10.0, 20.0 μg/mL) for 6 h, 12 h, or 24 h. After the supernatant was collected, cell viability was assessed by MTT [20]and DAPI staining.

Briefly, 20 μL of MTT (5 mg/mL) reagent was added to the cells and incubated at 37 °C for 4 h. The cell supernatant was removed, and 150 μL DMSO was added to dissolve the formazan crystals. After oscillation for 10min, the absorbance was measured with a microplate reader (Bio-tek, USA) under 570 nm.

After GES-1 cells were treated with purified rHpsh0459 for 12 h, the supernatant was discarded and washed with PBS at room temperature. Cells were stained with DAPI solution (Sigma) for 10 min at room temperature and subsequently examined by fluorescence microscopy (Olympus, Japan).

The supernatant of the cell proliferation assay (20 μg/mL rHpsh0459) was used to analyze IL-8 concentration. IL-8 levels were determined by ELISA (Invitrogen) according to the manufacturer's protocol.

2.6. Western blot analysis for protein-involved apoptosis

GES-1 cells were treated with four different concentration samples of rHpsh4590 (0.1, 2.5, 10.0, 20.0 μg/mL) for 6 h and the cells treated with PBS as control, then harvested to lyse with RIPA Lysis Buffer (Beyotime Institute of Biotechnology, China). Cell lysates were centrifuged at 13,000 rpm for 10 min, and the supernatants were collected to analyze cleaved caspase-3, cleaved PARP and GAPDH using Western blot.

2.7. Co-immunoprecipitation assay and mass spectrometry

Use the brucella broth medium supplemented with 5% fetal bovine serum (FBS) to collect the *H. pylori*. After the bacteria was lysis by 100 μL RIPA and 1 μL 10 mM PMSF, the100~500 μg total bacteria protein and 20 μL anti-Hpsh4590 antibody were incubated at 4 °C for 2 h, then 40 μL Protein A/G agarose was added and incubated at 4 °C for 2 h on a mixer. The rabbit polyclonal IgG to β-actin (Abcam, Cambridge, UK) was used as negative control. The immunoprecipitates were centrifuged at 2,500 g for 5min. After washing 4 times with PBS, the sediments were subjected to SDS-PAGE separation for MS analysis by the Instrumental Analysis Center of *Jiangsu* University.

2.8. Statistical analysis

Statistical calculations were performed using SPSS version 13.0 (SPSS, Chicago, USA). Measurement data were compared using Two-Factor ANOVA. *P*-values less than 0.05 were considered significant.

3. Results

3.1. Hpsh0459 might be a channel protein

The Hpsh4590 structure is based on computer modeling shows that, it comprises 411 amino acids with a molecular weight of 46431.4Da and belongs to the TrbI superfamily. Hpsh4590 is a hydrophilic protein, and the signal peptide was not calculated by SignalP 4.1. The potential antigenic epitopes are mainly distributed at the C-terminal, which was used to prepare the Hpsh4590 antibodies. The result of three-dimensional structure shows that