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The type II histidine triad protein HtpsC facilitates invasion of epithelial cells by highly virulent *Streptococcus suis* serotype 2[§]

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Streptococcus suis **serotype 2 (*S. suis* 2) is an important zoonotic pathogen that presents a significant threat both to pigs and to workers in the pork industry. The initial steps of *S. suis* 2 pathogenesis are unclear. In this study, we found that the type II histidine triad protein HtpsC from the highly virulent Chinese isolate 05ZYH33 is structurally similar to internalin A (InlA) from *Listeria monocytogenes*, which plays an important role in mediating listerial invasion of epithelial cells. To determine if HtpsC and InlA function similarly, an isogenic *htpsC* mutant (Δ*htpsC*) was generated in *S. suis* by homologous recombination. The *htpsC* deletion strain exhibited a diminished ability to adhere to and invade epithelial cells from different sources. Double immunofluorescence microscopy also revealed reduced survival of the Δ*htpsC* mutant after cocultivation with epithelium. Adhesion to epithelium and invasion by the wild type strain was inhibited by a monoclonal antibody against E-cadherin. In contrast, the *htpsC*-deficient mutant was unaffected by the same treatment, suggesting that E-cadherin is the host-cell receptor that interacts with HtpsC and facilitates bacterial internalization. Based on these results, we propose that HtpsC is involved in the process by which *S. suis* 2 penetrates host epithelial cells, and that this protein is an important virulence factor associated with cell adhesion and invasion.**

Keywords**:** *Streptococcus suis* serotype 2, HtpsC, invasion, adhesion, epithelial cells

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Introduction

Streptococcus suis 2 (*S. suis* 2) is an important zoonotic pathogen associated with a wide range of diseases in pigs, including meningitis, septicaemia, pneumonia, endocarditis, and arthritis (Staats *et al.*, 1997; Lun *et al.*, 2007). The organism can also be transmitted to humans via the respiratory or gastrointestinal tracts, or through contaminated wounds. Infection by *S. suis* 2 can cause septic arthritis, meningitis, endocarditis, and even streptococcal toxic shock-like syndrome (STSLS), manifested by a short course of disease accompanied by acute high fever, multiple organ failure, and high lethality (62.7–81.3%). The pathogen therefore poses a significant threat to public health (Normile, 2005; Sriskandan and Slater, 2006). Two major outbreaks of *S. suis* 2 have occurred in China (one in Jiangsu Province, 1998, and the other in Sichuan Province, 2005), suggesting that this infectious disease may emerge as a major global health issue (Tang *et al.*, 2006).

Genomic analyses and experimental studies have revealed that an ~89 kb pathogenicity island plays a critical role in the virulence of prevalent strains (Chen *et al.*, 2007; Shi *et al.*, 2016). In addition to this pathogenicity island, the discovery of a diverse group of virulence factors and regulators in past decades (Segura *et al.*, 2017) has greatly accelerated our understanding of *S. suis* 2 pathogenesis (Ye *et al.*, 2016; Zheng *et al.*, 2018). However, little is known about the mechanism by which *S. suis* 2 adheres to and invades mucosal epithelium cells to initiate an infection. These critical steps are required in order for the pathogen to defeat host defensive barriers, infect organs, and spread throughout the body. Although many virulence factors have been tentatively implicated in *S. suis* 2 adhesion, the experiments typically compared the ability of wild type strains and isogenic mutants to adhere to human laryngeal Hep-2 cells (Li *et al.*, 2011; Segura *et al.*, 2016). Importantly, most studies reported adhesion but in fact evaluated both adhesion and invasion because the assays combined intra- and extracellular bacteria. Although *S. suis* 2 is commonly regarded as an extracellular bacterium, it is capable of invading and surviving in host respiratory epithelial cells (Seitz *et al.*, 2013; Meng *et al.*, 2016), intestinal epithelial cells (Ferrando *et al.*, 2015; Ferrando and Schultsz, 2016), microvascular endothelial cells (Vanier *et al.*, 2004, 2007), choroid plexus epithelial cells (Schwerk *et al.*, 2012), astrocytes (Auger *et al.*, 2015; Liu *et al.*, 2017), and other cells. The interactions between *S. suis* 2 and host cells are therefore not understood in detail. The polysaccharide capsule is thought to hinder the adhesion and invasion of *S. suis* 2, but its actual role is still controversial (Benga *et al.*, 2010; Fittipaldi *et al.*, 2012). The cytotoxic effects of hemolysin appear to play an important role in host cell invasion by *S. suis* 2, but strains

Table 1. Bacterial strains, cell lines, and plasmids used in this study

Strains/plasmids	Description	Reference/source
S. suis 05ZYH33	Virulent strain isolated from a patient with STSLS	Lab collection
ΔhtpsC	Isogenic *htpsC* deletion mutant of *S. suis* strain 05ZYH33; Spc^r	This study
E. coli DH5α	Cloning host for recombinant plasmids	Sangon Biotech
pUCm-T	Cloning vector; Amp^r	Sangon Biotech
pUC18	Cloning vector; Amp^r	Lab collection
pUC18- *htpsC*	A recombinant vector with the background of pUC18 designed for knockout of *htpsC*; Amp^r; Spc^r	This study
pSET2	*E. coli-S. suis* shuttle vector, used to obtain the spectinomycin resistance cassette (Spc^r)	Takamatsu *et al.* (2001)
Hep-2	Human epidermoid cancer cells	Lab collection
A549	Human adenocarcinoma alveolar basal epithelial cells	Sangon Biotech
Caco-2	Human epithelial colorectal adenocarcinoma cells	Sangon Biotech

lacking hemolysin can still invade epithelial cells (Fittipaldi *et al.*, 2012; Meng *et al.*, 2016). These results suggest that other mechanisms may mediate *S. suis* invasion of host epithelial cells.

The highly pathogenic *S. suis* strain 05ZYH33 was isolated during an outbreak in Sichuan in 2005. Gene *SSU05-1577* in this strain encodes HtpsC, a type II histidine triad protein that functions in the adherence of *S. suis* 2 to Hep-2 cells and affects *S. suis* 2 virulence in mice (Li *et al.*, 2015). The relationship between HtpsC and bacterial invasion has not yet been elucidated. We found that HtpsC has high sequence similarity and shares a three-dimensional structure with internalin A (InlA) from *Listeria monocytogenes*. In food-borne infections caused by *L. monocytogenes*, InlA mediates recognition and invasion of epithelial cells through specific interactions with the host cell receptor E-cadherin, a transmembrane glycoprotein that connects epithelial cells at adherens junctions (Lecuit *et al.*, 2004; Bierne *et al.*, 2007; Pizarro-Cerdá *et al.*, 2012). Since the two proteins have similar features, we hypothesize that HtpsC plays a role in host cell invasion.

To confirm our conjecture, an *htpsC* deletion mutant was constructed in *S. suis* 2 by homologous recombination. Wild type and mutant versions of the strain were then compared in adhesion and invasion assays using three different sources of epithelial cells. Antibody inhibition assays were also used to explore whether E-cadherin has a role in the invasion process. Our results demonstrate that HtpsC functions in the internalization of *S. suis* by epithelial cells.

Materials and Methods

Bacterial strains, cell lines, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Streptococcus suis* 2 strains were grown at 37°C in Todd-Hewitt broth (THB; Hopebio) or on THB agar (Biotrading). *Escherichia coli* strain DH5α was grown in Luria Broth (LB) medium or plated on LB agar at 37°C. Antibiotics

Table 2. Primers used for PCR amplification and detection

Primers	Sequences (5′–3′)	Functions	Product lengths
HtpsC-A	TTACTCGCTTACCTTTGCTTCC	Resequence *htpsC*	2,436 bp
HtpsC-B	ATACGACTTGTAGTGTCTGAAAT		
LA-1	GGCCCTGCAGATTTATTTTCATTATT (*Pst* I)	Left arm of *htpsC*	1,338 bp
LA-2	CGGGATCCCTACTTTTGAGATATTGCTAC (*Bam*H I)		
Spc-1	CGGGATCCGTTCGTGAATACATGTTAT (*Bam*H I)	Resequence Spc^r	1,130 bp
Spc-2	CCGGGAGCTCGTTTTCTAAAATCTGAT (*Sac* I)		
RA-1	CCGGGAGCTCTTCTCATGTTTGGTAA (*Sac* I)	Right arm of *htpsC*	1,001 bp
RA-2	CCGGAATTCAATAACGGTGGAAATCT (*Eco*R I)		
NC-1	GTGACCAAATGGTTCTTGAC	Reference gene *gapdh* for RT-qPCR	261 bp
NC-2	ATTCAGTAGCAGCAGCTTTC		
RT-1	ATTTCCTTGTCCGTATTGC	Internal region of *htpsC* used for RT-qPCR	282 bp
RT-2	GAGGGACTGGTCTTAGAACATA		
Check-1A	AACCTGAAAATAGTCCCATAATA	Verification of the Δ*htpsC* mutant	1,377 bp for Δ*htpsC*； 2,483 bp for 05ZYH33
Check-1B	TCATTCAAGATGTTCAAAGTAGCA		
Check-2A	TTTGAAGGTCCGATCCCGTATG	Internal region of *htpsC* for verification	597 bp
Check-2B	CCCAGTATTTCCTTGTCCGTATTG		
Check-3A	TTACTCGCTTACCTTTGCTTCC	Verification of the Δ*htpsC* mutant	2,481 bp
Check-3B	TCATTCAAGATGTTCAAAGTAGCA		
1576-A	CAATGACACTCTTATCGCTCTT	Internal region of *SSU05-1576* used for RT-qPCR	249 bp
1576-B	CAGGTTGAGATGGCATTTAG		
1578-A	ACTCGTTGCGGAACAGAA	Internal region of *SSU05-1578* used for RT-qPCR	279 bp
1578-B	GCTTGTTGTAGCGGTGAA		

were used as follows: 100 μg/ml of spectinomycin (*Spc*) (Sigma) for *S. suis* transformants, and 50 μg/ml of ampicillin (*Amp*) (Sigma) for *E. coli.*

Hep-2 cells, A549 cells (Sangon Biotech), and Caco-2 cells (Sangon Biotech) were grown in an incubator at 37°C with an atmosphere adjusted to 5% CO_2. Hep-2 cells and A549 were grown in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS), while Caco-2 cells were grown in MEM (Sangon Biotech) supplemented with 20% FBS.

HtpsC sequence analysis

Sequence homology searches and conserved domain analyses were conducted using the Basic Local Alignment Search Tool (BLAST) on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST). Protein structure homology modeling was performed using SWISS-MODEL Workspace (http://swissmodel.expasy.org/) and visualized with the PyMOL viewer. Signal peptides were predicted using the SignalP 4.1 server.

Construction of the *S. suis* 2 *htpsC* deletion mutant

A recombination-based method was used to construct a mutation at the *htpsC* locus of *S. suis* 2 05ZYH33, as previously described (Li *et al.*, 2008). Briefly, a spectinomycin resistance cassette (Spc^r) was amplified by PCR from pSET2 and then inserted into a pUC18 vector to construct the recombinant plasmid pUC18-Spc^r. Two DNA fragments (LA and RA) flanking the *htpsC* gene were amplified from 05ZYH33 genomic DNA and cloned into pUC18-Spc^r to generate the knockout plasmid pUC-*htpsC*. The pUC-*htpsC* plasmid was then used for transformation of 05ZYH33 competent cells. Transformants were confirmed using multiplex-PCR assays with several specific primer pairs (Table 2), followed by direct DNA sequencing.

Cell adhesion and invasion assays

The assays were performed as described previously, with modifications (Maddocks *et al.*, 2013). Briefly, Hep-2, A549, and Caco-2 cells were seeded into 24-well plates containing culture medium and incubated at 37°C in a 5% CO_2 atmosphere for 48 h. *Streptococcus suis* strains (wild type and Δ*htpsC*) were grown in THB until reaching the exponential growth phase, then harvested by centrifugation for 5 min at 4,000 × *g*. Bacterial cells were washed three times with PBS, resuspended in DMEM, and 1.2×10^7 CFU of *S. suis* cells were used to infect the Hep-2, A549, and Caco-2 monolayers at a multiplicity of infection of 100 bacteria per epithelial cell (MOI = 100). The infected cells were incubated 2 h at 37°C to enable adhesion and internalization of bacteria. The monolayers were then washed three times with PBS to remove unbound bacteria. For adhesion assays, cells were treated with 0.5 ml of PBS containing 0.1% saponin, and lysed on ice for 15 min to release cell-associated (i.e., both extracellular and intracellular) bacteria. For invasion assays, infected cells were incubated for another 2 h in culture medium containing 100 μg/ml gentamicin to kill extracellular bacteria (internalized bacteria are spared). Cells were then washed twice in PBS and treated as described above to release internalized bacteria. To calculate the number of adherent bacteria, the number of internalized bacteria was subtracted from the total number of cell-associated bacteria. Assays were performed in triplicate and repeated at least three times.

Changes in transcription of *htpsC* before and after *S. suis* 2 invasion

Cell invasion assays followed the procedures described above. The cell lysates were collected, centrifuged, and resuspended. Bacterial RNA was extracted by TRIzol and reverse-transcribed to generate cDNA. The real-time PCR assay was performed using SYBR Green I chimeric fluorescence. The housekeeping gene *gapdh* served as an internal reference. The $2^{-\Delta\Delta CT}$ method was use to evaluate changes in *htpsC* transcript levels before and after *S. suis* invasion (Livak and Schmittgen, 2001).

Double immunofluorescence microscopy assays

Confocal double immunofluorescence microscopy was conducted as previously described, with the following modifications (Benga *et al.*, 2010). Hep-2, A549, and Caco-2 cells were added to a 24-well plate containing a cover glass to grow patches. Cells on coverslips were rinsed once with cell culture medium without antibiotics, and the cells were infected with a suspension of *S. suis* (WT or mutant strain) at an MOI of 100:1. After incubation for 2 h, cells were washed three times with PBS and fixed for 5 min with 4% paraformaldehyde. Formaldehyde-fixed preparations were washed and incubated for 20 min with PBS containing 1% FBS (blocking buffer) to block non-specific binding sites. Blocking buffer was removed and preparations were incubated with serum obtained from pigs previously infected with *S. suis* (1:100) for 45 min. After washing away the blocking buffer, preparations were incubated with rhodamine-conjugated goat anti-pig IgG antiserum (1:100) for 30 min to stain extracellular bacteria. Subsequently, preparations were washed three times with blocking buffer then acetone was used to permeabilize the epithelial cells for 15 min at -20°C. The preparations were again incubated with infected pig serum (1:100) for 45 min, washed with blocking buffer three times, and then incubated with FITC-conjugated goat anti-pig IgG antiserum (1:100) for 30 min to stain intracellular bacteria. Finally, to stain nuclei, DAPI was added and the preparations were incubated at room temperature in the dark for 5 min, then washed with blocking buffer 4 times. After the final washing step, the preparations were mounted with anti-fluorescence quencher and stored at 4°C until examination.

Anti-E-cadherin antibody blocking assays

Before *S. suis* 2 infection, Hep-2, A549, and Caco-2 cells were incubated overnight at 37°C in a 24 well plate with 100 μg/ml mouse anti-human E-cadherin monoclonal antibodies. Cell adherence and invasion experiments were performed as described above.

Statistical analysis

All data are expressed as means ± standard deviations (SD) (error bars). Differences were analyzed using a two-tailed, unpaired *t*-test. $P < 0.05$ was considered significant, and $P < 0.01$ was considered highly significant.

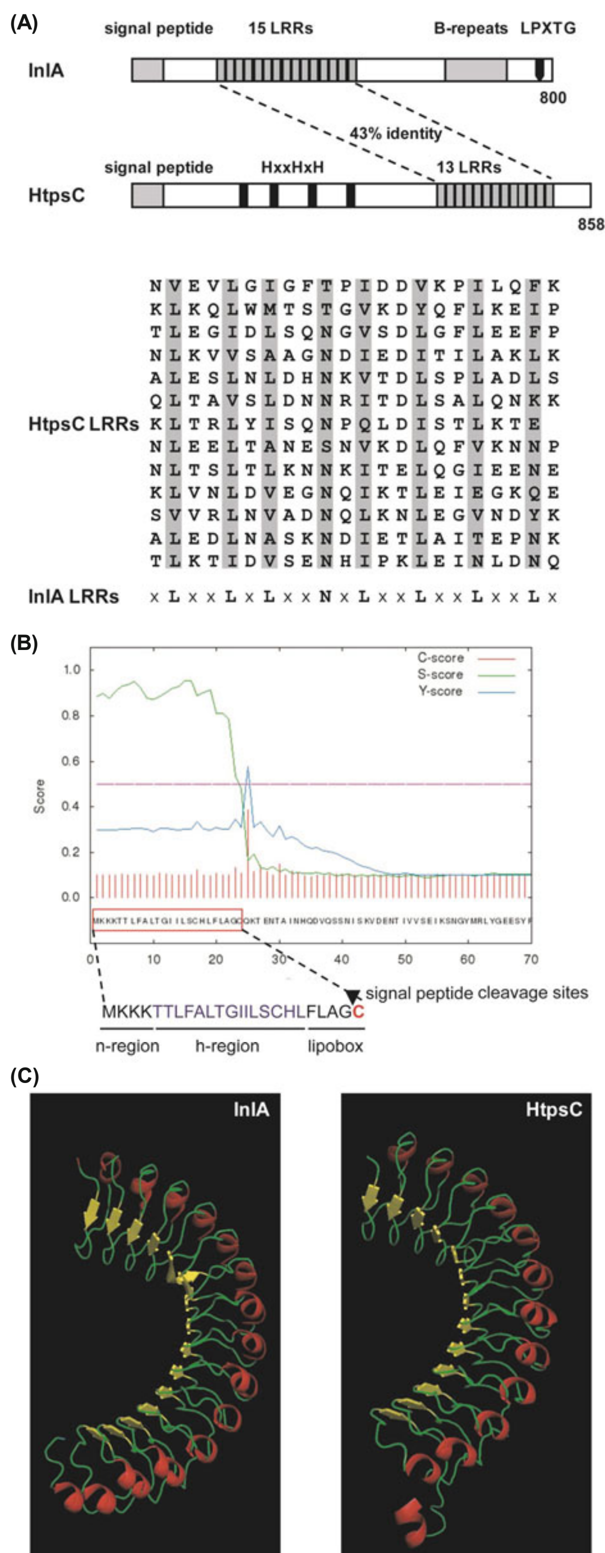

Fig. 1. **HtpsC sequence analysis.** (A) The top panel shows an overview of the domain architectures of InlA and HtpsC. Conserved LRR sequences are connected by dotted lines. The bottom panel shows an alignment of the 15 LRR regions in HtpsC and the LRR consensus from InlA. "x" represents any amino acid. (B) Prediction of HtpsC signal peptide. Signal sequence subdomains are shown in expanded view at the bottom. (C) Three-dimensional structure derived by homology modeling for LRRs from HtpsC and InlA.

Results

Comparison of molecular structures of InlA from *L. monocytogenes* and HtpsC from *S. suis*

As shown in Fig. 1A, the HtpsC precursor protein in *S. suis* strain 05ZYH33 contains 858 amino acid residues, including 13 leucine-rich repeats (LRRs) at the C-terminus. The LRRs share 45% amino acid identity with the corresponding region in InlA. A potential signal sequence characteristic of a surface lipoprotein is present at the N-terminus, followed by 4 histidine trimer domains (HxxHxH). As predicted by SignalP 4.1, the lipoprotein signal peptide sequence consists of the first 24 amino acid residues of the HtpsC precursor (Fig. 1B), beginning with an n-region containing positively-charged residues, followed by a hydrophobic h-region, and finally a conserved c-region (known as the lipobox) preceding the lipidated cysteine.

After homologous modeling, the three-dimensional structural model of the LRR domains of HtpsC closely match the solved crystal structure of InlA (Fig. 1C). Both present similar horseshoe-shaped configurations, suggesting that HtpsC and InlA may have similar biological functions.

Construction and characterization of an isogenic *htpsC* mutant

Using homologous recombination, the *htpsC* gene was successfully replaced by the *Spc*^{r} cassette in a double cross-over event (Fig. 2A). The Δ*htpsC* mutant was verified by PCR analysis with different several primer pairs (Fig. 2B) and by direct sequencing. The *htpsC* deletion has no obvious effect on the growth of *S. suis* 2 (Fig. 2C).

Contribution of HtpsC to bacterial adhesion and invasion

Streptococcus suis Δ*htpsC* has a significantly reduced ability to adhere to and invade Hep-2, A549, and Caco-2 cells (Fig. 3), suggesting that HtpsC has an important role in the initial steps of infection. *S. suis* 2 adheres most strongly to Hep-2 cells, but invades Caco-2 cells at higher levels than the other two cell lines.

Expression of *htpsC* increases after cell invasion

htpsC mRNA levels were compared before and after *S. suis* invasion of epithelial cells. The results show that expression of *htpsC* is up-regulated significantly after invasion of Hep-2, A549, and Caco-2 cells (1.44-fold, 2.45-fold, and 2.06-fold, respectively) (Fig. 4).

Determination of cell adherence and invasion by double immunofluorescence

Immunofluorescence double staining was used to compare Δ*htpsC* and wild type *S. suis* in adhesion and invasion assays. The number of Δ*htpsC* adhering to and invading cells was significantly lower than for wild type *S. suis* in Hep-2, A549, and Caco-2 cell lines (Fig. 5). The results are consistent with those shown in Fig. 3 and support the conclusion that the *htpsC* deletion significantly affects *S. suis* adhesion to and invasion of epithelial cells. The wild type strain showed stronger invasiveness vs. Caco-2 than to the other two cell lines, which again is consistent with the experiment described previously.

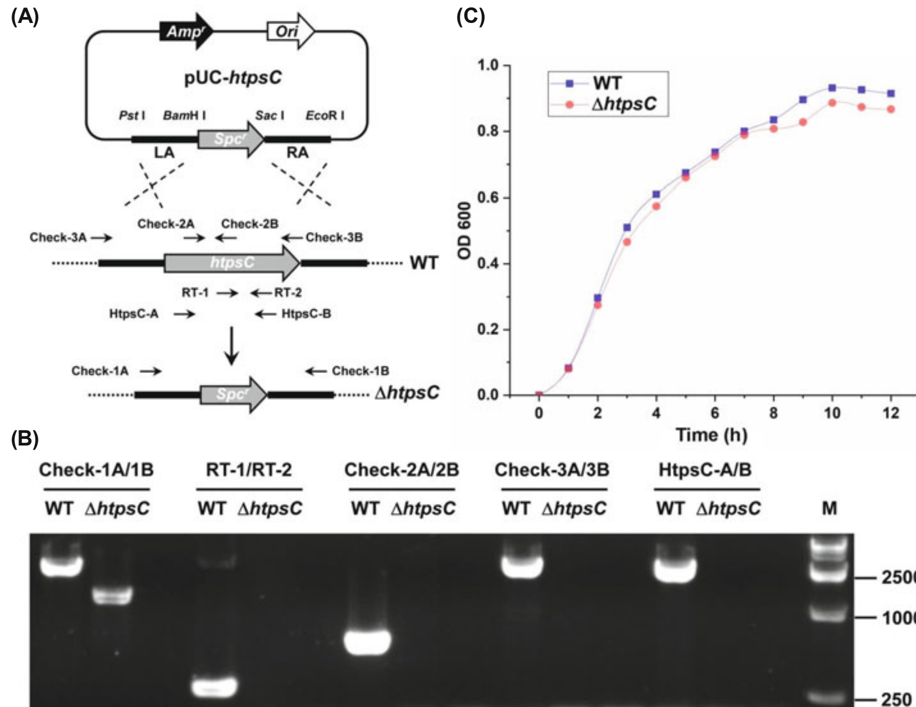

Fig. 2. **Construction of an isogenic *htpsC* mutant of *S. suis* 05ZYH33.** (A) Knockout strategy for replacement of *S. suis* 2 *htpsC* by a *Spc*^{r} cassette. LA and RA are the left and right boundaries of the target gene, respectively. (B) Multiplex PCR analysis of the Δ*htpsC* mutant. The PCR products were separated by 1.0% agarose gel electrophoresis and stained with ethidium bromide. The corresponding primer pairs are shown in Table 2. (C) Growth curves for cultures of *S. suis* 2 wild type and Δ*htpsC* strains. Culture density was measured by spectroscopy at 600 nm.

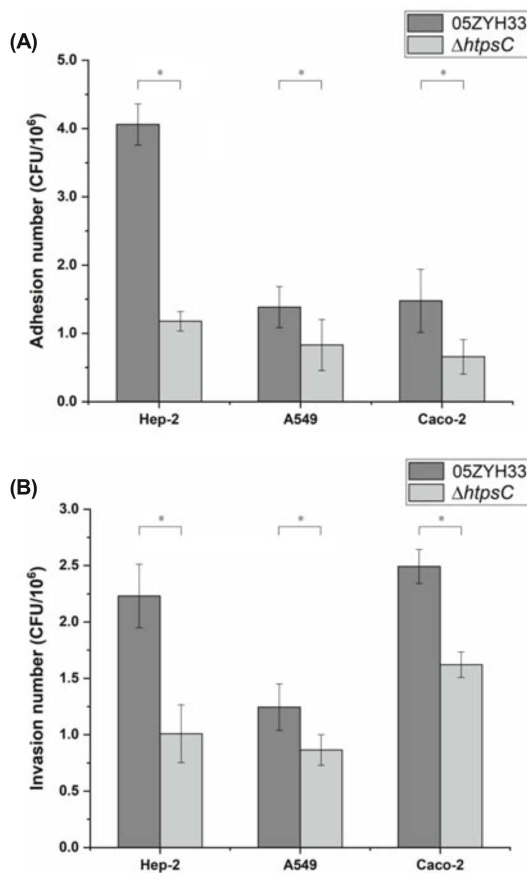

Fig. 3. **Deletion of *htpsC* significantly reduces adhesion to and invasion of epithelial cells by *S. suis* 2.** (A) Comparison of adhesion of wild type and Δ*htpsC* mutant strains. (B) Comparison of invasion of the wild type and Δ*htpsC* mutant strains.

Anti-E-cadherin antibody inhibition assays

Anti-E-cadherin antibody blocking significantly impaired the ability of wild type *S. suis* 2 to invade epithelial cells, compared to untreated cells (Fig. 6). In contrast, invasion by the Δ*htpsC* mutant was not affected by antibody blocking. These results suggest that interaction between the E-cadherin receptor and HtpsC is an important step in the invasion of epithelial cells by *S. suis* 2.

Discussion

In this study, we found that HtpsC in the Chinese epidemic

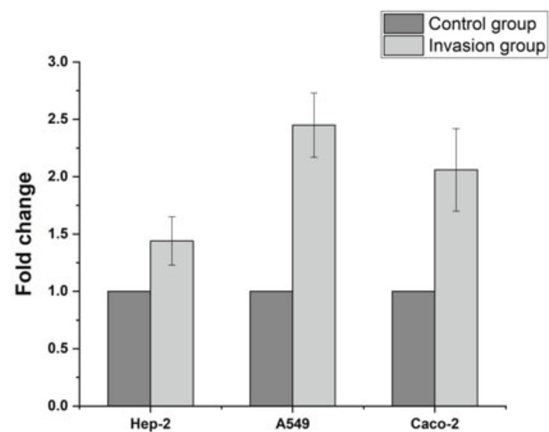

Fig. 4. **Transcription of *htpsC* before and after invasion.** After invading epithelial cells, expression of *htpsC* was significantly up-regulated. Values have been normalized to *gapdh* expression, and wild-type expression level is defined as 1.

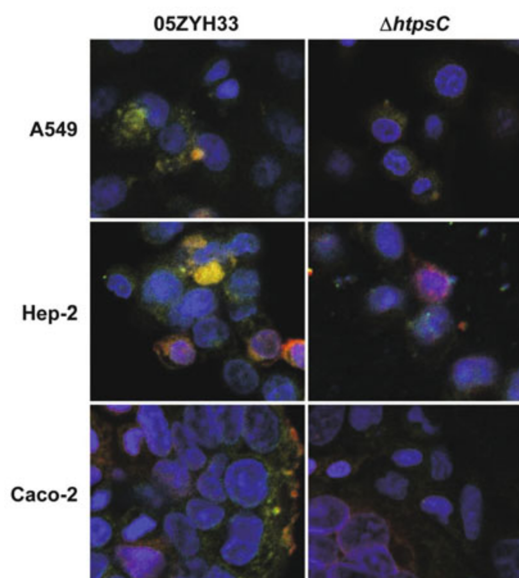

Fig. 5. **Determination of extra- and intracellular bacteria by double immunofluorescence (DIF) after incubation of *S. suis* 2 strains with epithelial cells.** Cell lines A549, Caco-2, and Hep-2 were infected with strain 05ZYH33 or the Δ*htpsC* mutant. Extracellular bacteria are stained orange-red and intracellular bacteria are green (× 840). Nuclei were labelled by DAPI (blue).

strain *S. suis* 05ZYH33 plays a critical role in enabling the pathogen to enter epithelial cells. Although there are differences in structural organization between HtpsC and listerial InlA, these two proteins may have similar functions. The C-terminal LPXTG motif of InlA is recognized by sortase A, which cleaves the motif and anchors InlA to the bacterial cell wall (Garandeau *et al.*, 2002). The 15 LRRs at the InlA N-terminus extend from the bacterial cell wall to enable binding to the surface receptor of a target cell. HtpsC is also expressed on the cell surface of *S. suis* 2 (Li *et al.*, 2015). However, since lipoprotein signal peptides contribute significantly to cell surface protein localization in Gram-positive bacteria (Nielsen *et al.*, 1997; Sutcliffe and Harrington, 2002), we speculate that the N-terminal lipoprotein signal peptide of HtpsC plays an important role in the anchoring process, while the 13 LRRs at its C-terminus protrude from cell wall to facilitate receptor binding. It has been well-documented that LRRs are versatile binding motifs optimized for protein-protein interactions and are found in a variety of proteins involved in signal transduction (Hamon *et al.*, 2006), bacterial adhesins (Bober *et al.*, 2011), and internalin or immune receptors (e.g. Toll-like and NOD-like receptors) in mammals (Ng *et al.*, 2011) and plants (Zipfel and Felix, 2005). There is a large disparity in the number of genes that encode LRR proteins in pathogenic and other bacterial types (Fouts *et al.*, 2016). For ex-

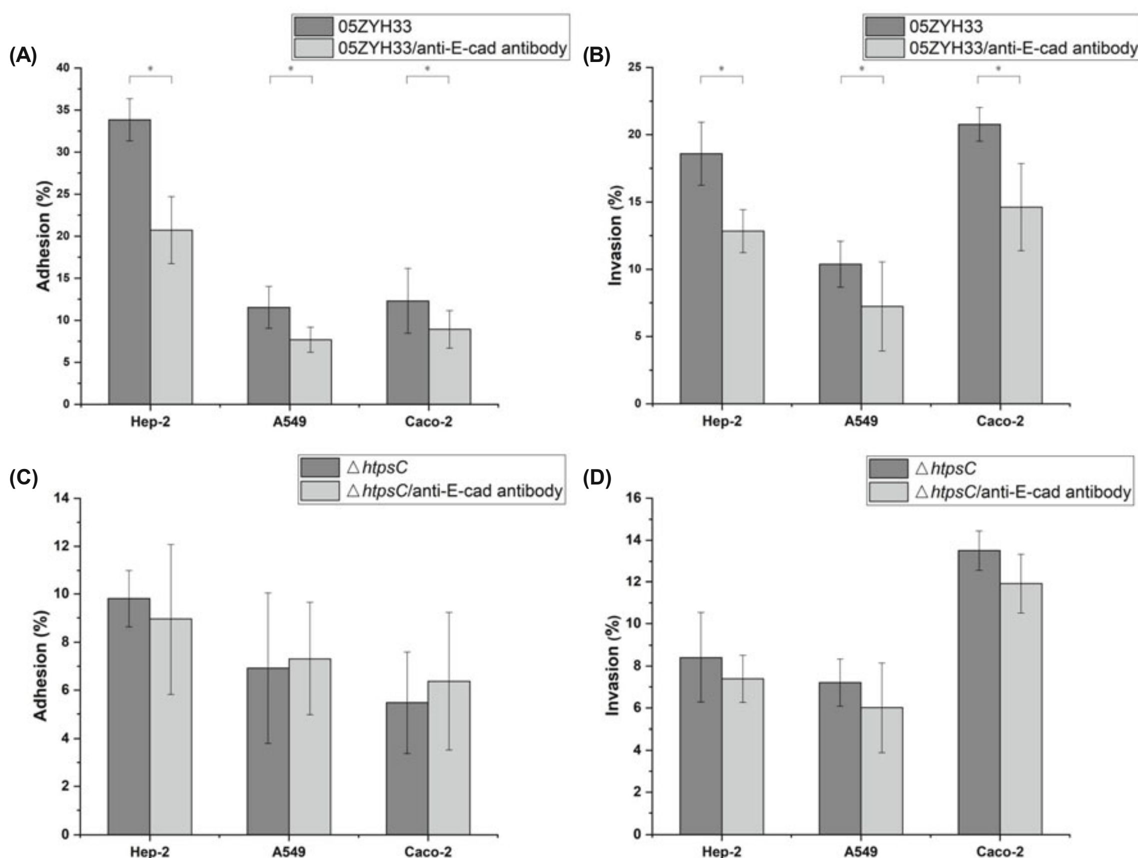

Fig. 6. **Anti-E-cadherin antibody blocks HtpsC-mediated internalization of *S. suis* 2.** (A and B) Comparison of adhesion and invasion by *S. suis* wild type strain 05ZYH33 vs. epithelial cells before and after E-cadherin monoclonal antibody treatment. (C and D) Comparison of adhesion and invasion by *S. suis* Δ*htpsC vs*. epithelial cells before and after E-cadherin monoclonal antibody treatment.

ample, the pathogen *Leptospira interrogans* encodes at least 20 LRR-containing proteins, whereas the nonpathogenic *Leptospira biflexa* encodes only one annotated LRR protein (Picardeau, 2017), suggesting that LRR-containing proteins are important for bacterial pathogenesis.

To learn more about HtpsC function, we performed a transcriptional analysis and found that *htpsC* is induced *in vivo* at the cellular level, consistent with results obtained by *in vivo* induced antigen technology (IVIAT) (Li *et al.*, 2013). Genes which are specifically expressed or up-regulated *in vivo* (designated *in vivo* induced genes) may contribute to *in vivo* growth and pathogen virulence (Handfield *et al.*, 2005). We generated a *htpsC* deletion mutant to further analyze the contribution of HtpsC to cell adhesion and invasion of *S. suis*, but have not yet constructed a revertant for testing. However, the genes flanking *htpsC* in the mutant strain are expressed at normal levels (Supplementary data Fig. S1), indicating that the *htpsC* knockout has not introduced any detectable polar effects.

We found that the ability of *S. suis* 2 to adhere to epithelial cells was similar to previous reports. However, the wild type strain 05ZYH33 exhibits unusually high invasion rates of 18.6%, 10.4%, and 20.7% in Hep-2, A549, and Caco-2 cell lines, respectively. These results contrast with previous studies that showed *S. suis* 2 to be poorly invasive (Bennett-Wood *et al.*, 1998; Lalonde *et al.*, 2000). Lalonde *et al.* (2000) examined three *S. suis* 2 virulent strains isolated from Europe and North America and reported that *S. suis* 2 adheres to but does not invade epithelial cells. Using a strain isolated from a newborn piglet, Seitz *et al.* (2013) concluded that suilysin promotes adherence and invasion of non-encapsulated *S. suis* 2 in Hep-2 epithelial cells. Nevertheless, the invasion rate was below 0.2% (Normile, 2005). In studies of host-pathogen interactions between the intestinal mucosa and *S. suis* 2, Ferrando *et al.* (2015) found that nearly all *S. suis* 2 strains tested invaded Caco-2 cells poorly. A possible explanation for the discrepancy is that the strain used in our study (05ZYH33), isolated from a Chinese patient with STSLS, is highly virulent by virtue of a distinctive pathogenicity island acquired by horizontal gene transfer. Bacterial virulence might correlate well with the ability to invade epithelium, as has been reported in group B (GBS) and group A streptococci (GAS) (Valenti-Weigand *et al.*, 1996; Bennett-Wood *et al.*, 1998). In those studies, the ability to invade Hep-2 cells is strongly indicative of the relative virulence of the strains and the clinical outcome of disease following infection, while adherence to Hep-2 epithelial cells and virulence does not always correlate. Norton *et al.* (1999) also found that virulent strains enter more Hep-2 cells than do less virulent strains. Moreover, the virulent bacteria were found in higher numbers per Hep-2 cell than the less virulent strains.

Another important result from our present study is that the ability of the Δ*htpsC* mutant to invade epithelial cells is diminished significantly. Since it was known that inactivation of *htpsC* decreases adherence of *S. suis* 2 to Hep-2 cells (Li *et al.*, 2015), we focused on the function of HtpsC during the internalization process. Antibody inhibition experiments using anti-E-cadherin reveal that interaction between HtpsC and E-cadherin may be essential for *S. suis* adhesion and entry into epithelial cells. The interaction between InlA and E-cadherin involves the N-terminal region of InlA, which encompasses the LRR region and the inter-repeat (IR) region. Briefly, this structure is necessary and sufficient to promote bacterial entry into cells expressing the corresponding receptor. The LRR region interacts directly with E-cadherin, whereas the IR region is required for proper folding of the LRR region (Lecuit *et al.*, 1997). Miras *et al.* (2015) solved the crystal structure of an extracellular *L. interrogans* LRR protein and identified a binding pocket that facilitates the binding of human E-cadherins and host-pathogen interactions (Eshghi *et al.*, 2018). Based on these findings, we hypothesize that the LRR domain contributes substantively to the interaction between HtpsC and E-cadherin. Interestingly, the N-terminal HTP domain and the C-terminal LRR domain of HtpsC contribute independently to HtpsC adherence to fibronectin and laminin, which are two extracellular matrix proteins located on the Hep-2 cell surface (Li *et al.*, 2015). More experiments will be required to verify whether the HTP or LRR domains contribute to the interaction between HtpsC and E-cadherin.

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Conflict of Interest

The authors have no conflict of interest to report.

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