Gene 603 (2017) 42–53

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Deletion of the *vacJ* gene affects the biology and virulence in *Haemophilus parasuis* serovar 5

Liangyou Zhao [a,b], Xueli Gao [a], Chaonan Liu [a], Xiaoping Lv [a], Nan Jiang [c], Shimin Zheng [a,*]

[a] College of Veterinary Medicine, Northeast Agricultural University, Harbin 150030, People's Republic of China
[b] Drug Safety Evaluation Center of Heilongjiang University of Chinese Medicine, Harbin 150040, People's Republic of China
[c] College of Life Science and Technology, Dalian University, Dalian 116622, People's Republic of China

ARTICLE INFO

Article history:
Received 12 June 2016
Received in revised form 28 October 2016
Accepted 10 December 2016
Available online 14 December 2016

Keywords:
Haemophilus parasuis
VacJ lipoprotein
Outer membrane integrity
Biofilm formation
Serum resistance
Adherence and invasion
Virulence

ABSTRACT

Haemophilus parasuis is an important pathogen causing severe infections in pigs. However, the specific bacterial factors that participate in pathogenic process are poorly understood. VacJ protein is a recently discovered outer membrane lipoprotein that relates to virulence in several pathogens. To characterize the function of the *vacJ* gene in *H. parasuis* virulent strain HS49, a *vacJ* gene-deletion mutant Δ*vacJ* and its complemented strain were constructed. Our findings supported that VacJ is essential for maintenance of cellular integrity and stress tolerance of *H. parasuis*, by the demonstrations that the Δ*vacJ* mutant showed morphological change, increased NPN fluorescence and, and decreased resistance to SDS-EDTA, osmotic and oxidation pressure. The increased susceptibility to several antibiotics in the Δ*vacJ* mutant further suggested that the stability of the outer membrane was impaired as a result of the mutation in the *vacJ* gene. Compared to the wild-type strain, the Δ*vacJ* mutant strain caused a decreased survival ratio from the serum and complement killing, and exhibited a significant decrease ability to adhere to and invade PK-15 cell. In addition, the Δ*vacJ* mutant showed reduced biofilm formation compared to the wild-type strain. Furthermore, the Δ*vacJ* was attenuated in a murine (Balb/C) model of infection and its LD_{50} value was approximately fifteen-fold higher than that of the wild-type or complementation strain. The data obtained in this study indicate that *vacJ* plays an essential role in maintaining outer membrane integrity, stress tolerance, biofilm formation, serum resistance, and adherence to and invasion of host cells related to *H. parasuis* and further suggest a putative role of VacJ lipoprotein in virulence regulation.

1. Introduction

Haemophilus parasuis, a member of the family Pasteurellaceae, is one of the most important bacteria affecting pigs. It is the causative agent of Glässer's disease, which is characterized by fibrinous polyserositis, polyarthritis and meningitis (Oliveira and Pijoan, 2004). This pathogen is considered as an early colonizer and a member of the normal microbiota of the upper respiratory tract of piglets (Møller and Kilian, 1990). However, virulent strains can invade hosts and cause severe systemic disease under certain conditions. *H. parasuis* infection produces significant morbidity and mortality in contemporary swine production systems, and giving rise to large economic losses in swine-rearing countries (Cerdà-Cuéllar et al., 2010).

Multiple different genotypes and serotypes of *H. parasuis* have been described (Kielstein and Rapp-Gabrielson, 1992). However, there is not a clear association between virulence and *H. parasuis* phenotypes or genotypes, and the knowledge of the pathogenesis and putative virulence factors mechanisms in *H. parasuis* are largely unknown (Olvera et al., 2007; Chen et al., 2012). Virulence factor is a material basis of bacterial virulence which plays a major role in the pathogenesis of the pathogen. So the study of the biological characteristics of virulence factor has become the primary task of the pathogenic mechanism. However, very few virulence-associated factors have been identified in *H. parasuis* to date (Costa-Hurtado and Aragon, 2013; Blackall and Turni, 2013).

Outer membrane lipoproteins are widely distributed in Gram-negative bacteria which are involved in diverse mechanisms of physiology/pathogenesis (Sutcliffe et al., 2012). The VacJ lipoprotein was initially discovered in *Shigella flexneri* and is attributed for bacterial spreading (Suzuki et al., 1994). A previous study demonstrated that the *vacJ* gene of Non-typeable *Haemophilus influenzae* has contributed to serum resistance and IgM binding, thus mediating the escape from complement-dependent killing (Nakamura et al., 2011). VacJ is widely distributed among members of Pasteurellaceae and several other Gram-

Abbreviations: NAD, nicotinamide-adenine dinucleotide; LB, Luria-Bertani; TSA, Tryptic Soy Agar; TSB, Tryptic Soy Broth; OD_{600}, optical density at 600 nm; Kan, kanamycin; MIC, minimal inhibitory concentration; LD_{50}, 50% lethal dose; i.p., intraperitoneally; ORF, open reading frame.

* Corresponding author.
E-mail address: zhengshiminbl@sohu.com (S. Zheng).

http://dx.doi.org/10.1016/j.gene.2016.12.009
0378-1119/

negative bacterial species, however, the functional characterization in the pathogenicity of *H. parasuis* has not been determined till date.

H. parasuis stain SH0165 gene HAPS_2270 has also been predicted to encode VacJ lipoprotein (Yue et al., 2009). In this study, we inactivated the *vacJ* gene in *H. parasuis* serovar 5 strain HS49 and investigated the biological characteristics of *vacJ* mutant strain, including growth characteristics, outer membrane integrity, biofilm formation, serum resistance, adhesion and invasion to preliminary understand the role of *vacJ* gene in *H. parasuis*. Then the virulence of the Δ*vacJ* mutant was tested in mice to determine the effect of *vacJ* gene in virulence of *H. parasuis*.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultured in Luria-Bertani (LB) medium. *H. parasuis* was plated on Tryptic Soy Agar (TSA) or grown in Tryptic Soy Broth (TSB) (Difco Laboratories, Detroit, MI, USA), supplemented with 0.01% nicotinamide adenine dinucleotide (NAD) (Sigma, St. Louis, MO, USA) and 10% inactivated bovine serum at 37 °C. If necessary, 50 μg/mL of kanamycin or 20 μg/mL of gentamicin was complemented. To measure bacteria growth curves, cultures of bacteria were grown overnight in TSB supplemented with NAD and serum. The cultures were subinoculated into fresh TSB medium at a ratio of 1:100 and incubated at 37 °C. The optical density at 600 nm (OD_{600}) was measured at 1 h intervals.

2.2. Prediction of VacJ characteristics and lipobox motif

The subcellular location of VacJ lipoprotein was predicted using the Cell-Ploc package (http://chou.med.harvard.edu/bioinf/Cell-PLoc/) (Chou and Shen, 2008). The VacJ characteristics were predicted with the PROTEAN program as well as proteomics tools from the ExPASy website (Shivachandra et al., 2014). The lipobox sequence in VacJ was predicted using the DOLOP program (Babu et al., 2006). The *vacJ* sequence was analyzed for identity and similarity to known sequences using BLAST (http://www.ncbi.nlm. nih.gov/BLAST/). Multiple alignment analyses of VacJ protein sequences were performed using ClustalX.

Table 1
Bacterial strains and plasmids.

Strains and plasmids	Relevant characteristics	Source
E. coli DH5α	FΦ80ΔlacZΔM15Δ(lacZYA-argF) U169 recA1 endA1 hsdR17	Laboratory collection
H. parasuis		
HS49	serotype 5 field isolate	Laboratory collection
Δ*vacJ* (HS49Δ*vacJ::kan*)	*vacJ* mutant of *H. parasuis* HS49, Kan^R	This work
C-*vacJ*	The complement of *H. parasuis* HS49 Δ*vacJ::kan* containing pK18-C-*vacJ*, Kan^R, Gm^R	This work
Plasmids		
pK18mobsacB	Suicide and narrow-broad-host vector, Kan^R	Schafer et al. (1994)
pK18-Δ*vacJ*	A 949 bp fragment containing the upstream and downstream sequences of the *vacJ* gene in pk18mobsacB, Kan^R	This work
pK18-Δ*vacJ::kan*	A 1884 bp fragment containing Δ*vacJ::kan* cassette in pk18mobsacB, Kan^R	This work
pK18-C-*vacJ*	A 2496 bp fragment containing *vacJ::Gm* cassette in pk18mobsacB, Kan^R Gm^R	This work
pBAD18-Km	Km resistance cassette-carrying vector, Kan^R	Guzman et al. (1995)
p34s-Gm	Gm resistance cassette-carrying vector, Gm^R	Yamanaka et al. (1995)

2.3. Construction of H. parasuis ΔvacJ mutant and complemented strains

Primers used for amplification (Table 2) in this study were ordered from the Invitrogen (Shanghai, China). A 949 bp PCR fragment containing the 488 bp upstream of the ATG start codon and the 461 bp downstream of the TAA stop codon of the *vacJ* gene was amplified using overlap extension PCR with primers (P1 and P4), and subsequently cloned into plasmid pK18mobsacB to generate the plasmid pK18-Δ*vacJ*. Both sets of primers (P1 and P4) contained a 9 bp core DNA uptake signal sequence (Zhang et al., 2011). A kanamycin resistance cassette (935 bp) was amplified from pBAD18-Km using primers P5 and P6. Then the pK18-Δ*vacJ* and the kanamycin resistance cassette were digested with *Bam*HI and *Sal*I and ligated together to create plasmid pK18-Δ*vacJ::kan*. The recombinant plasmid was introduced into *H. parasuis* strain HS49 by natural transformation, as described previously (Zhang et al., 2011, 2012).

The complementation strain C-*vacJ* was constructed according to the method of Saeed-Kothe et al. (2004) and Zhang et al. (2012). A PCR fragment was amplified using overlap PCR with primers (P7 and P10), which contained the complete open reading frame (ORF) of *vacJ* gene and the gentamicin resistance cassette. Both the fragment and the pK18-Δ*vacJ* plasmid were excised with *Bam*HI and *Sal*I and then ligated together to form a single-copy, chromosome-based complementation plasmid of pK18-C-*vacJ*, and then this plasmid was transformed into the Δ*vacJ* mutant. The gentamicin-resistant transformants were checked for specified homologous recombination by PCR with primers P7 and P10.

2.4. Growth characteristics

The *H. parasuis* wild-type strain HS49, mutant strain Δ*vacJ*, and C-*vacJ* were cultured in 5 mL TSB supplemented with 10% inactivated bovine serum and 0.01% NAD for 16 h, and then diluted to OD_{600} of 1.0. The fresh cultures were then grown in 50 mL of the same medium at 37 °C. Samples of culture were monitored at 1 h intervals using a Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany). Uninoculated TSB served as the blank control. The colony forming units (CFUs) were determined by performing a dilution series from the culture and counting colonies from the appropriate dilution at 2 h intervals.

2.5. Transmission electron microscopy

For morphological detection, the bacteria were grown to logarithmic phase in TSB and harvested by centrifugation. Pellets were resuspended in phosphate buffered saline (PBS) and washed three times. Then the cultures were fixed and dehydrated as described previously (Murphy et al., 2006) to be detected by transmission electron microscopy (TEM).

2.6. SDS-EDTA permeability assay

SDS-EDTA permeability assay was conducted as described previously by Carpenter et al. (2014). Briefly, the *H. parasuis* wild-type strain

Table 2
Sequences of PCR oligonucleotide primers.

Primers	Sequence (5′–3′)[a]
P1 (*vacJ*-up-F)	CGCAAGCTT*ACCGCTTGT*GCAGAAATTGTTGATTACTC
P2 (*vacJ*-up-R)	GTCGACATGCTCGGATCCATGACTACTTTAAAAAAGGG
P3 (*vacJ*-down-F)	GGATCCGAGCATGTCGACTGATTTACCTGTAAAAATTT
P4 (*vacJ*-down-R)	CGGAATTC*ACAAGCGGTT*CACTCGATTCTTTAATGCG
P5 (Kan-F)	CGGGATCCGTAAGGTTGGGAAGCCCTGC
P6 (Kan-R)	CGCGTCGACGGTCGGTCATTTCGAACCCC
P7 (*vacJ*-F)	CGCGGATCCATGAAAAAAATTAAACTTCT
P8 (*vacJ*-R)	CAACCTTACTTACCATAATCAATCAATATTT
P9(Gm-F)	TTATGGTAAGTAAGGTTGCGAATTGACATAAG
P10(Gm-R)	ACGTGTCGACGAAGCCGATCTCGGCTTGAAC

[a] Restriction sites are underlined, uptake signal sequences (USS) are in italics.