Antonie van Leeuwenhoek (2017) 110:1647–1657
DOI 10.1007/s10482-017-0914-7

ORIGINAL PAPER

Polyamine-binding protein PotD2 is required for stress tolerance and virulence in *Actinobacillus pleuropneumoniae*

Zhuang Zhu · Qin Zhao · Yu Zhao · Fei Zhang · Xintian Wen · Xiaobo Huang · Yiping Wen · Rui Wu · Qigui Yan · Yong Huang · Xiaoping Ma · Xinfeng Han · Sanjie Cao

Received: 24 May 2017 / Accepted: 15 July 2017 / Published online: 21 July 2017
© Springer International Publishing AG 2017

Abstract *Actinobacillus pleuropneumoniae* is the cause of porcine contagious pleuropneumonia, which is one of the most important respiratory diseases in swine and causes huge economic losses in the swine industry. PotD, a polyamine-binding protein, has been well characterised in many pathogens of humans and animals. In this study, a *ΔpotD2* mutant of *A. pleuropneumoniae* strain MS71 (serovar 1) was constructed successfully by homologous recombination. Growth curves of different strains showed that the growth of the *ΔpotD2* mutant was affected greatly in the logarithmic phase compared with that of parental strain. In vitro stress assays revealed that the viability of *ΔpotD2* mutant strain was significantly impaired under multiple environmental stresses, including high temperature, oxidation and hyperosmosis. Additionally, the *ΔpotD2* mutant caused significantly decreased mortality in a mouse model. Taken together, the findings in this study suggest an important role of PotD2 in the growth, stress tolerance and virulence of *A. pleuropneumoniae*.

Keywords *Actinobacillus pleuropneumoniae* · *potD2* gene · Stress tolerance · Virulence

Zhuang Zhu, Qin Zhao, and Yu Zhao have contributed equally to this work.

Z. Zhu · Q. Zhao · Y. Zhao · F. Zhang · X. Wen · X. Huang · Y. Wen · R. Wu · Q. Yan · Y. Huang · X. Ma · X. Han · S. Cao (✉)
Research Center of Swine Disease, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, China
e-mail: veterinary226@163.com

X. Wen · X. Huang · Y. Wen · R. Wu · Q. Yan · S. Cao
Sichuan Science-Observation Experiment of Veterinary Drugs and Veterinary Biological Technology, Ministry of Agriculture, Chengdu, China

S. Cao
National Teaching and Experiment Center of Animal, Sichuan Agricultural University, Chengdu 611130, China

Introduction

Actinobacillus pleuropneumoniae is the causative agent of porcine contagious pleuropneumonia, which often causes serious fibrino-hemorrhagic necrotizing pneumoniae and fibrinous pleurisy in swine (Bossé et al. 2002). To date, *A. pleuropneumoniae* has been divided into 15 serotypes on the basis of the antigenic properties of the capsular polysaccharides and lipopolysaccharides and 2 biotypes according to the dependence on nicotinamide adenine dinucleotide (NAD) (Blackall et al. 2002; Zhang et al. 2016a). Although many studies have concentrated on *A. pleuropneumoniae*, porcine contagious pleuropneumonia is still one of the most serious disease in the swine industry worldwide (Chiers et al. 2010; Hillen et al. 2014).

Polyamines are a group of small polycationic compounds with a hydrocarbon backbone and

multiple amino groups, including putrescine, spermidine, spermine, and cadaverine (Nasrallah et al. 2014). Since they have the fully protonated state in physiological conditions, polyamines are positively charged and usually interact with intracellular negatively charged compounds, such as nucleic acids, ATP and phospholipids (Miyamoto et al. 1993; Childs et al. 2003). Because of their interactions with other compounds, polyamines have affected many aspects of cellular physiology, and play an important role in cell growth, stabilising biological membrane structure, stress tolerance and signaling (Igarashi and Kashiwagi 2010). Moreover, many studies have shown that polyamines are involved in the expression of virulence factors (Durand and Björk 2003), immune evasion (Bussière et al. 2005; Chaturvedi et al. 2010), biofilm formation (Karatan et al. 2005; Patel et al. 2006) and oxidative stress resistance in several pathogens (Chattopadhyay et al. 2003).

Polyamines can be obtained by autosynthesis or uptake from the extracellular environment in most bacteria. Currently, polyamine transport systems are widely studied in several human pathogens, which covers five types of uptake pathway. Spermidine-preferential uptake system PotABCD and putrescine-specific transport systems PotFGHI are typical ATP-binding cassette (ABC) transporters, in which ATP are needed to provide energy. In addition, two antiporter systems, the PotE-mediated putrescine-ornithine antiporter and CadB-mediated cadaverine-lysine antiporter, and a putrescine-specific uniporter PuuP, have also been identified in *Escherichia coli* (Kashiwagi et al. 1992; Soksawatmaekhin et al. 2004; Kurihara et al. 2005, 2009). Among these, the PotABCD system is the most widely distributed polyamine transport system, in which PotA is a cytoplasmic protein with an ATP-binding motif that couples ATP hydrolysis to provide energy for translocating polyamines. Two polypeptides PotB and PotC form a transmembrane channel through which extracellular polyamines are transported. PotD is a periplasmic substrate-binding protein that binds extracellular polyamine (Kashiwagi et al. 1990; Pistocchi et al. 1993; Igarashi et al. 2001; Shah and Swiatlo 2008).

It has been identified that a putative polyamine-binding protein PotD2 is present in all 15 serovars of *A. pleuropneumoniae* (Liao et al. 2009; Chung et al. 2012). However, it is still unknown what influence the biological characteristics of PotD2 may exert in *A. pleuropneumoniae*. In this study, an unmarked *ΔpotD2* mutant as well as the corresponding genetically complemented strain C-*ΔpotD2* were constructed to investigate the possible biological functions of PotD2 in *A. pleuropneumoniae.*

Materials and methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are described in Table 1. Wild-type *A. pleuropneumoniae* strain MS71 (serovar 1), an isolate from a diseased pig in Sichuan, China, was used as the parental strain. MS71 and its derivatives were grown in Tryptic Soy Broth (TSB, BD, USA) or on Tryptic Soy Agar (TSA, BD, USA) supplemented with 5% bovine serum (Invitrogen, USA) and 0.01% NAD. When necessary, the media were supplemented with 50 μg/ml kanamycin or 5 μg/ml chloramphenicol (Cm). *Escherichia coli* strains were grown on Luria–Bertani (LB, Difco Laboratories, Detroit, USA) agar or in broth. When required, the media were supplemented with 25 μg/ml chloramphenicol and 1 mM diaminopimelic acid (Sigma-Aldrich, St. Louis, MO, USA). All strains were grown at 37 °C.

Bioinformatic analysis

The amino acid sequence of the putative protein PotD2 of *A. pleuropneumoniae* serovar 1 str.4074 was obtained from the NCBI (Protein ID = WP005596365.1). ProtParam (http://www.expasy.org/) was used to compute the pI/Mw value. The signal peptide domain, secondary structure, hydrophobicity, and subcellular location were predicted by the programs LipoP (http://www.cbs.dtu.dk/services/LipoP/), Pred-Lipo (http://www.compgen.org/tools/PRED-LIPO), Psipred (http://bioinf.cs.ucl.ac.uk/psipred/), ProtScale (http://web.expasy.org/protscale/) and Psort (http://psort1.hgc.jp/form.html), respectively.

Construction of the *ΔpotD2* mutant in *A. pleuropneumoniae*

The primers used for amplification are listed in Table 2. The *ΔpotD2* mutant was constructed as described previously (Li et al. 2016). Briefly,

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source
Strain		
MS71	*APP* serovar 1 clinical isolate	Collection
△*potD2*	Unmarked potD2-negative deletion mutant of MS71	This study
C-△*potD2*	MS71 complemented △*potD2* strain	This study
E. coli DH5a	Cloning host for maintaining the recombinant plasmids	Takara
E. coli BL21 (DE3)	Expressing host for maintaining the recombinant plasmids	Takara
E. coli β2155	thrB1004 pro thi hsdS lacZΔM15 (F' lacZΔM15 lacI^q traD36 proA$^+$ proB$^+$) Δdap::erm (Erm^r)	Baltes et al. (2003)
Plasmid		
pEMOC2	Transconjugation vector based on pBluescript SK with mobRP4, a polycloning site, Cm^r, and transcriptional fusion of the *omlA* promotor with the sacB gene	Baltes et al. (2003)
pLS88	Broad-host-range shuttle vector from *Haemophilus ducreyi*; Str^r Kan^r	Willson et al. (1989)
pET32a(+)	Expression vector, Amp^r	Takara
pLR15	Upstream and downstream homologous fragment of potD2 cloned into pEMOC2	This study
pCP	Containing *potD2* gene in pLS88, Kan^r	This study

approximately 1 kb upstream and downstream homologous arms of *potD2* were amplified using the strain MS71 genome as template by primers potD2-Up-F/R and potD2-Down-F/R, respectively. The two PCR fragments were integrated by overlap extension PCR using primers potD2-Up-F and potD2-Down-R. The resulting PCR product was digested by SalI and NotI,and subsequently inserted into the suicide plasmid pEMOC2 which was also digested by SalI and NotI to generate the recombinant suicide plasmid pLR15. The resulting pLR15 was conjugated into strain MS71 using the *E. coli* strain β2155. After two homologous recombination steps, the *ΔpotD2* mutant was identified by PCR and sequencing assays (Fig. 1).

Construction of the complemented strain C-*ΔpotD2*

For creation of the genetically complemented strain, the entire open reading frame as well as the promoter region of *potD2* was cloned into the shuttle vector pLS88 to generate the expression plasmid pCP by homologous recombinant cloning. The resulting pCP was electroporated into the *ΔpotD2* mutant and the

Table 2 Sequences of PCR oligonucleotide primers

Primers	Sequence (5′–3′)[a]
potD2-UpF-Sal I	*CAAGTCGAC AAGTGAGTAAATGAGGGAGC*
potD2-UpR	*actttgcagggcttcccaaccttacTTAAGTTTTCTCCGTTAAAG*
potD2-DownF	*actctggggttcgaaatgaccgaccATTCGTTAGCCTAACGGATA*
potD2-DownR-Not I	*TGCGGCCGC GTGCCGCCTGATGACTTG*
potD2-F-BamH I	*CGGGATCC GAAGAAAAGCCAAAAGCGG*
potD2-R-HindIII	*CCCAAGCTT TTATTTCGCCGCTTTTAAC*
C-ΔpotD2-F	*tgaaagaaaccgcaagaattcTAATATAGCGTTAAATTTGCTGATTTCA*
C-ΔpotD2-R	*gtcacgctctccagcgagctcCGTGAGTTTAAATAGTTTACGTGTGTT*
ApxIV-F	*GCCATCGACTCAACCAT*
ApxIV-R	*TGGCACTGACGTGATGA*

[a] Restriction sites are underlined. The extended sequence required for overlap PCR and homologous recombinant cloning are indicated in minuscule

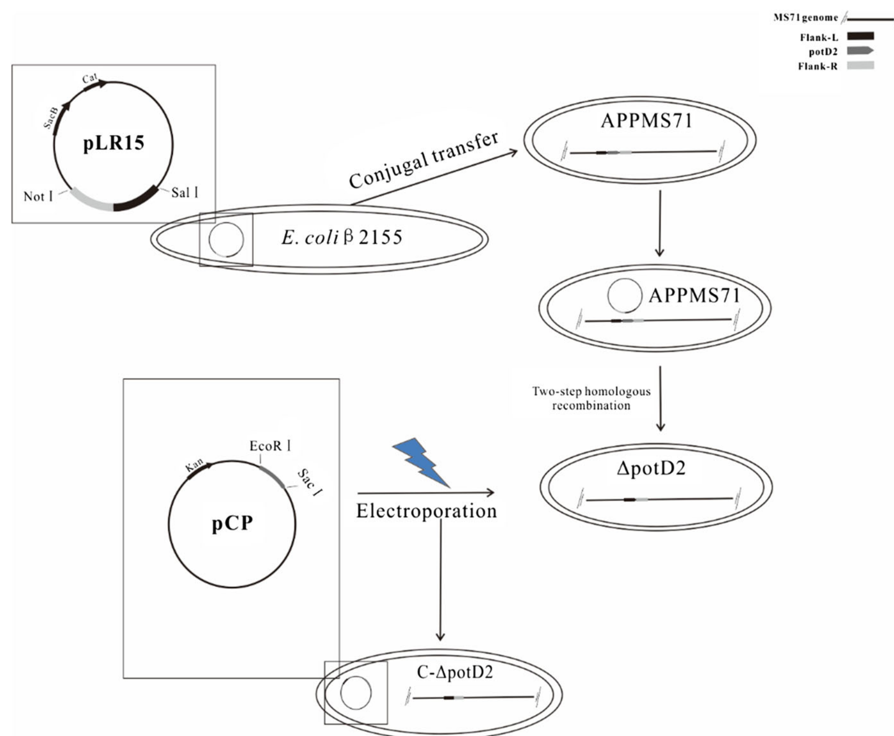


Fig. 1 Schematic representation of the construction of the *A. pleuropneumoniae ΔpotD2* mutant and the corresponding genetically complemented strain *C-ΔpotD2*

transformants were selected on TSA containing kanamycin (Fig. 1).

Expression of recombinant PotD2 and generation of mouse antisera

Recombinant PotD2 protein was generated using an *E. coli* expression system. Briefly, PCR fragments containing *potD2* gene minus its signal peptide sequence was amplified from MS71 genomic DNA using primers potD2-F/R. The resulting PCR products was then inserted into the BamHI and HindIII sites of pET32a to generate recombinant plasmid pET32a-PotD2. The resulting plasmids were transformed into *E. coli* BL21(DE3) and induced to express by the addition of 1 mM isopropyl-beta-D-thiogalactopyranoside. Recombinant proteins were purified by Ni affinity chromatography using Mini IMAC Ni-Charged column (Bio-Rad) as described previously (Zhang et al. 2016b).

To prepare the PotD2 antiserum, five female specific pathogen free mice (18–22 g) were immunised subcutaneously with 50 μg recombinant PotD2 protein emulsified in 100 μL complete Freund's adjuvant. 2 weeks following the initial immunisation, the mice were given a booster shot of the same dose of PotD2 protein and incomplete Freund's adjuvant. Serum samples were collected 2 weeks after booster immunisation and stored at −80 °C.

Western blotting

For western blotting analysis, the whole cell extract of strains MS71, *ΔpotD2* mutant and *C-ΔpotD2* complemented strain were analysed by 12% SDS-PAGE and electrotransferred to a polyvinylidene fluoride membrane. After being blocked with 5% skimmed milk in TBST (Tris–HCl buffered saline containing 0.05% Tween 20) at room temperature for 1 h, the membrane was incubated at room temperature for 2 h with mice antiserum against recombinant PotD2 protein as the primary antibody. Horseradish peroxidase-conjugated goat anti-mouse IgG (Bioss, China) were used as the secondary antibody. The membrane was developed with an Immun-Star Western C Kit (Bio-Rad, USA) according to the manufacturer's instructions.

Genetic stability and in vitro growth assays

The *ΔpotD2* mutant was cultured and passaged for 10 times continuously in TSB. The corresponding fragments were amplified by PCR to identify the genetic stability of each generation of the mutant strains. The single colonies of MS71, *ΔpotD2* mutant and *C-ΔpotD2* were picked respectively and inoculated in TSB with shaking overnight. The next day, the bacterial suspensions were inoculated into fresh TSB at a dilution of 1:100. The bacterial cells were incubated in TSB at 37 °C with 200 rpm/min. The optical density at 600 nm (A600) was measured at 1 h intervals. All experiments were performed in triplicate.

Stress tolerance assays

In vitro stress assays were conducted with minor modifications as described previously using the MS71 strain, *ΔpotD2* mutant and *C-ΔpotD2* strain (Fang et al. 2013). At an OD600 value of approximately 0.6, cells from 1 ml cultures were centrifuged at $5000\times g$ for 5 min. For the oxidative tolerance assay, the cells were resuspended in 1 ml of TSB supplemented with 0.8 mM hydrogen peroxide for 30 min. For the heat-shock assay, cells were resuspended in TSB and placed in a 50 °C water bath for 20 min. The control samples of each strain were resuspended in 1 ml TSB without any treatment. Then, the cultures from each stress resistance assay were serially diluted in TSB, and spread on TSA plates for CFU counting. For the osmotic tolerance assay, 1 ml cultures of each strain were diluted in TSB and then spread on TSA plates supplemented with or without 0.3 M NaCl or KCl for overnight incubation. Stress resistance was calculated as [(stressed sample CFU ml^{-1})/(control sample CFU ml^{-1})] $\times$ 100. The experiments were carried out in triplicate.

Virulence assays

To assess the virulence of strains MS71, *ΔpotD2* mutant and *C-ΔpotD2*, the Bliss method was used to measure the LD50 of each strain, respectively. For each strain, seventy 6-week-old specific pathogen free mice (Sichuan Province Dossy Experimental Animal Centre, Chengdu, China) were allocated to seven groups, each group containing ten mice, half of male and female. According to the pre-experiment measured LD0 and LD100 values, the mice were injected intraperitoneally with a group dose of geometrically diluted MS71, *ΔpotD2* and *C-ΔpotD2* strains, respectively. All surviving mice were euthanized 7 days post infection.

Statistical analysis

The statistical significance of the data was determined using Student's *t* test with SPSS19.0. For all data, a value of $p < 0.05$ was considered as significance.

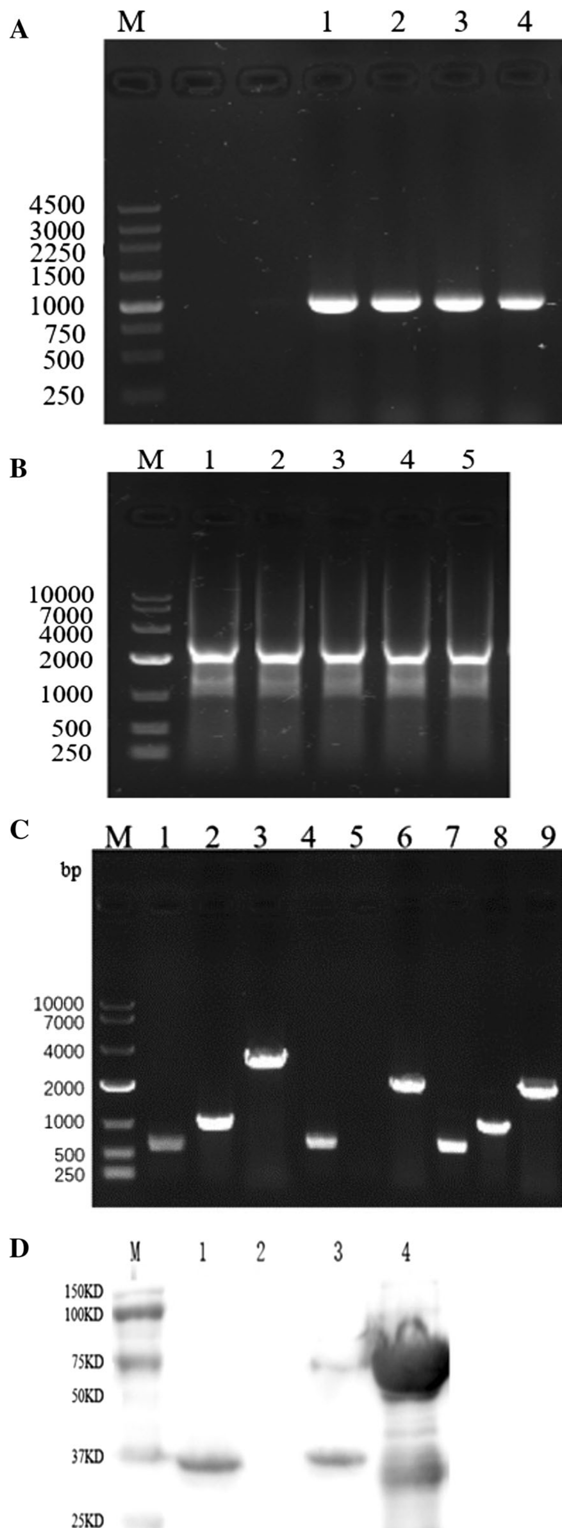
Results

Bioinformatics analysis of PotD2 protein in *A. pleuropneumoniae*

To provide some insights into the understanding of the biological functions of PotD2 protein in *A. pleuropneumoniae*, multiple bioinformatics tools were employed to analyse the structure and physicochemical properties of the PotD2 protein. Intriguingly, PotD2 has a type II signal peptide containing an $LTAC_{20}N$ 'lipobox' motif and is thus predicted to be a lipoprotein. As N_{21} would act as a Lol avoidance signal (Hara et al. 2003), we predict PotD2 to be an inner membrane lipoprotein interacting with the PotB/PotC permease complex. The GRAVY value of PotD2 calculated by ProtParam got −0.334, which suggested PotD2 a hydrophilic protein. Secondary structure analysis by Predict Protein and Psipred showed that the alpha helix, extended strand and random coil occupied 41, 12 and 47% of the sequence, respectively.

Generation of a *ΔpotD2* mutant and its complemented strain *C-ΔpotD2*

The upstream and downstream homologous arms were successfully amplified (Fig. 2a) and integrated (Fig. 2b). The *ΔpotD2* mutant was preliminary confirmed by PCR: the *potD2* fragment (1083 bp) was amplified from both MS71 and *C-ΔpotD2* strains using primers potD2-F/R, but not in the *ΔpotD2* mutant (Fig. 2c). The 3151 bp fragment (upstream-potD2-downstream) was amplified from the MS71 strain while both *ΔpotD2* mutant and *C-ΔpotD2* strain



◀**Fig. 2** **a** The PCR amplification of homologous arms. *Lane1–2* Upstream homologous arms, *Lane3–4* Downstream homologous arms; *Lane M* shows DNA molecular marker. **b** The upstream and downstream homologous arms were integrated by overlap extension PCR (*Lane 1–5*). **c** PCR identification of the *potD2* deletion and complementation. Primers potD2-F/R were used to amplify the *potD2* gene fragment from MS71 strain (*Lane 2*), *ΔpotD2* strain (*Lane 5*) and C-*ΔpotD2* strain (*Lane 8*); primers potD2-UD-F/R were used to amplify *potD2* upstream to downstream from MS71 strain (*Lane 3*), *ΔpotD2* strain (*Lane 6*) and C-*ΔpotD2* strain (*Lane 9*); primers ApxIV-F/R were used to amplify the ApxIV gene fragment from MS71 strain (*Lane 1*), *ΔpotD2* strain (*Lane 4*) and C-*ΔpotD2* strain (*Lane 9*). **d** Western blotting analysis of the MS71 strain, *ΔpotD2* strain and C-*ΔpotD2* strain. The lysates of the MS71, *ΔpotD2* and C-*ΔpotD2* strains were detected using anti-PotD2 antibodies. The C-*ΔpotD2* strain displayed the similar band as that of the parent strain MS71, while *ΔpotD2* strain did not. *Lane 1* MS71 strain, *Lane 2 ΔpotD2* strain, *Lane 3* C-*ΔpotD2* strain, *Lane 4* positive control (PotD2 protein)

yielded a 2068 bp fragment (upstream–downstream) using the primers potD2-UD-F/R (Fig. 2c). Additionally, western blotting assays further showed that the PotD2 protein was detected in the lysates of strains MS71 and *C-ΔpotD2* but not in lysate of the *ΔpotD2* strain (Fig. 2d). Together, these results indicated the successful generation of a *potD2* gene deletion mutant and its complemented strain.

Growth characteristics of the *ΔpotD2* mutant

Compared with the parent strain MS71, the growth curve of the *ΔpotD2* mutant showed a decrease in the logarithmic phase. However, both MS71 and *ΔpotD2* mutant could achieve the same growth level after entry into the stationary phase. The growth ability of the *C-ΔpotD2* and MS71 strains remained at a comparable level (Fig. 3). The results showed that the deletion of *potD2* gene led to growth defects in the logarithmic phase, while this delay effect was overcome by stationary phase.

PotD2 is required for the stress tolerance of *A. pleuropneumoniae*

The parental strain MS71, *ΔpotD2* mutant and complemented strain *C-ΔpotD2* were exposed to various stress conditions. When the cells were treated with 0.8 mM hydrogen peroxide for 30 min, the survival rate of *ΔpotD2* mutant was 9%, which was significantly lower than that of the MS71 (74%) and *C-*

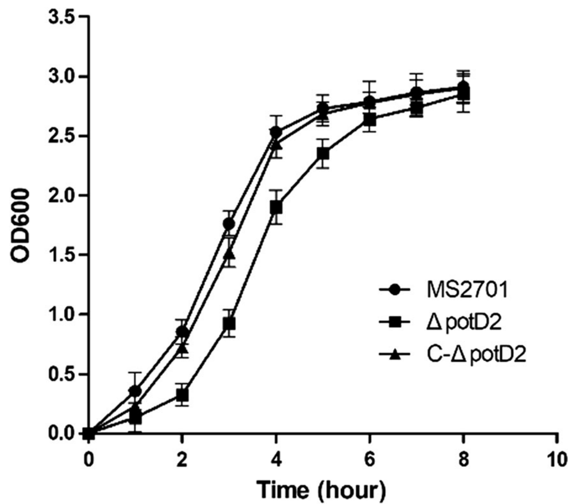


Fig. 3 Growth curves of the parent strain MS71, *ΔpotD2* mutant and genetically complemented strain *C-ΔpotD2* in TSB broth

ΔpotD2 strains (67%; Fig. 4a). This result suggested that the PotD2 protein plays an important role in the tolerance of oxidative stress in *A. pleuropneumoniae*. Similar results were obtained in the heat shock assay. After incubation at 50 °C water for 20 min, the parental strain MS71 exhibited an 91% survival rate, whereas only 49% of the *ΔpotD2* mutant cells survived. The survival ability of the complemented strain *C-ΔpotD2* was comparable with the MS71 strain (83%) (Fig. 4b). These results indicated that the deletion of *potD2* impaired the ability of *A. pleuropneumoniae* to respond to heat shock. In the osmotic tolerance assay, after exposure to 0.3 M NaCl or KCl, the survival rate of *ΔpotD2* mutant cells (66%/62%) was significantly lower than that of the MS71 (87/85%) and *C-ΔpotD2* (83%/81%) strains (Fig. 4c), indicating that the PotD2 is also important for the response of *A. pleuropneumoniae* to osmotic stress. Taken together, these results indicate that the PotD2 protein is involved in the tolerance of oxidative, heat and osmotic stresses in *A. pleuropneumoniae*.

Attenuation of the *ΔpotD2* mutant in mice

The LD50 values of strains MS71, *ΔpotD2* and *C-ΔpotD2* are listed in Table 3. Compared with the LD50 of the parent strain MS71, the *ΔpotD2* mutant showed an approximately 1.5-fold decrease, while the complemented strain exhibited a comparable

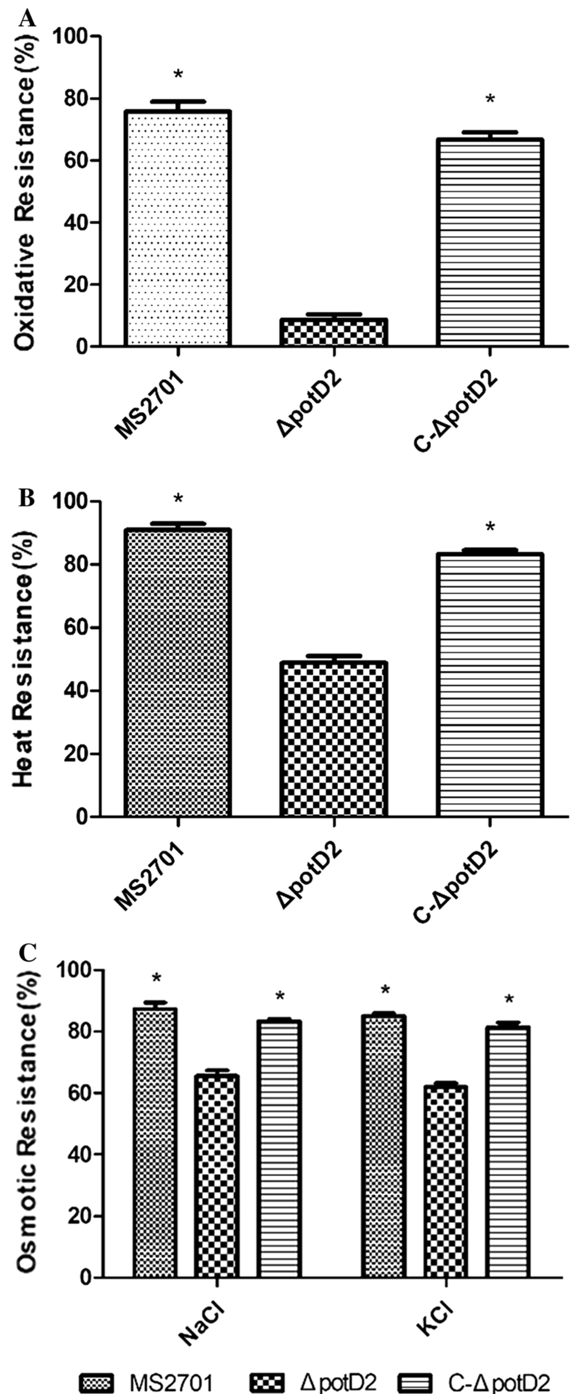


Fig. 4 Impaired stress tolerance of the *ΔpotD2* mutant. Overnight cultures were inoculated into fresh medium and grown to an OD600 value of approximately 0.6. Cells were then treated with **a** 0.8 mM H_2O_2 for 30 min, *p $<$ 0.001, **b** 50 °C heat shock for 20 min, *p $<$ 0.01, **c** 0.3 M NaCl or KCl, *p $<$ 0.01. Asterisk denotes p values (*t* test) for comparison to *ΔpotD2*

virulence. The results showed that the deletion of *potD2* gene led to an significant attenuation of *A. pleuropneumoniae*.

Discussion

PotABCD is a conserved extracellular polyamine transport system that is present in many bacteria and the periplasmic PotD protein is responsible for binding polyamines prior to transportation. A putative *potD2* homologue is present in all 15 serotypes of *A. pleuropneumoniae*: in this study, we successfully constructed an unmarked *ΔpotD2* mutant and corresponding genetically complemented strain, aiming to investigate the role of PotD2 in *A. pleuropneumoniae*.

To date, the specific structure and function of the PotD2 protein in *A. pleuropneumoniae* is still unclear. Therefore, bioinformatics tools were employed to predict and analyse the structure of PotD2 protein of *A. pleuropneumoniae*. Surprisingly a lipoprotein signal peptide was detected in the PotD2 with a cleavage site between position A19 and C20, along with an N21 Lol avoidance signal, suggesting that the protein is transported as a precursor protein through the cell membrane, lipid modified and retained at the inner membrane. The proportion of hydrophilic amino acids was higher than hydrophobic amino acids in PotD2 protein. This implied that overall this protein was more likely to be hydrophilic, which is consistent with the GRAVY value of PotD2 calculated by ProtParam. PotD2 exhibits 54% (175/322) amino acid identity with the PotD protein of *E. coli* which has been structurally characterised (PDB 1POY; Sugiyama et al. 1996a, b).

Based on the result of growth curves of the parent strain and the *ΔpotD2* mutant, we found that PotD2 may play a role in the logarithmic growth phase but does not affect the overall growth of *A. pleuropneumoniae*. Previous studies (Nasrallah et al. 2014; Yodsang et al. 2014) have shown that the deletion of *potD* gene did not affect the growth of several bacterial species under normal conditions. We conclude that the deletion of the *potD2* gene may lead to the defects of extracellular polyamine transportation in the early period of growth, resulting in a reduced polyamine concentration in *A. pleuropneumoniae* that is eventually overcome, presumably due to a redundancy of polyamine transport systems.

Table 3 Virulence of the MS71 strains, ΔpotD2 mutants and C-ΔpotD2 strains in mice

Strains	Challenge dose[a] (CFU)	Dead/total	Death rate (%)	Values of LD^{b}_{50}
MS71	1.2×10^7	10/10	100	$LD50 = 4.6 \times 10^6$
	8.4×10^6	8/10	80	
	5.4×10^6	6/10	60	
	3.6×10^6	4/10	40	
	2.4×10^6	1/10	10	
	1.6×10^6	0/10	0	
	PBS control	0/10	0	
ΔpotD2	2.2×10^7	10/10	100	$LD50 = 6.9 \times 10^6$
	1.4×10^7	9/10	90	
	9.0×10^6	7/10	70	
	5.8×10^6	3/10	30	
	3.7×10^6	2/10	20	
	2.4×10^6	0/10	0	
	PBS control	0/10	0	
C-ΔpotD2	1.4×10^7	10/10	100	$LD50 = 5.0 \times 10^6$
	9.3×10^6	8/10	80	
	6.1×10^6	6/10	60	
	4.0×10^6	4/10	40	
	2.7×10^6	2/10	20	
	1.8×10^6	0/10	0	
	PBS control	0/10	0	

[a] Groups of seven SPF mice were injected intraperitoneal with 200 μL of bacterial suspension containing various quantities of MS71, ΔpotD2 and C-ΔpotD2, respectively. The number of survivors was recorded until 7 days

[b] LD50 calculated by the method of Bliss

To assess the role of PotD2 in resistance to various hostile environments in vitro, the survival rate of the *ΔpotD2* mutant was determined. The results showed that the *ΔpotD2* mutant exhibited significantly reduced tolerance of heat shock, hyperosmosis, and especially oxidation. As previous studies showed that polyamines play a role in directly removing intracellular reactive oxygen species (Rider et al. 2007), we suggest that a reduced polyamine concentration in the *ΔpotD2* mutant lowered the ability to remove reactive oxygen species, which ultimately caused damage to the bacterial cells. In addition, studies in *E. coli* showed that the concentration of polyamines was associated with the expression of OxyR that regulates the expression of superoxide dismutase and hydrogen peroxidase, both of which can protect *E. coli* from oxidation (Tkachenko and Nesterova 2003; Chiang and Schellhorn 2012). We speculate that the expression of APL_RS07810, a homologous gene of *OxyR* in *A. pleuropneumoniae*, may be suppressed in the *ΔpotD2* mutant, leading to a downregulation in the expression of superoxide dismutase and hydrogen peroxidase. In the heat-shock assays, the results showed that the *ΔpotD2* mutant was defective in the tolerance of high temperature. This result is in accordance with previous studies in *Streptococcus pneumoniae*, in which the transcriptional level of the *potD* gene was higher both in lower and higher temperature conditions than in normal culture conditions (Shah et al. 2008). In the osmotic assays, the *ΔpotD2* mutant exhibited greater sensitivity to osmotic stress than the parental strain, indicating that PotD2 aids tolerance to osmotic stress in *A. pleuropneumoniae*. Previous studies suggested two feasible mechanisms by which polyamines may contribute to the osmotic resistance. Firstly, polyamines are involved in the regulation of extracellular and intracellular osmotic pressure as osmolytes (Schiller et al. 2000). Secondly, in combination with some bacterial porins, such as OmpC and OmpF, polyamines can act as endogenous regulators of outer membrane permeability. As a result, polyamines play a role in the suppression of channel opening and the enhancement of channel closure, as well as the promotion of blocked or inactivated states (Vega and Delcour 1996; Iyer and Delcour 1997).

In the virulence studies, we observed that the *ΔpotD2* mutant was attenuated compared with the parental strain MS71 and complemented strain *C-ΔpotD2* in mice, suggesting that PotD2 may be involved in the virulence of *A. pleuropneumoniae*. However, as mouse models cannot reflect the exact infection process of *A. pleuropneumoniae* in the natural host, further studies should be performed to evaluate the virulence of these strains using porcine model.

Overall, our findings in this study demonstrated that PotD2 plays a demonstrable role in tolerance of multiple stresses, particularly oxidation stress. We found that the PotD2 is required for optimal growth of *A. pleuropneumoniae* in the logarithmic phase. Moreover, we showed that PotD2 contributes to the full virulence of *A. pleuropneumoniae* infection in mice. Further studies are needed to determine the underlying mechanisms of PotD2 activity and its significance in stress tolerance of *A. pleuropneumoniae*.

Acknowledgements This research was financially supported by a grant from the Special Fund for Agro-Scientific Research in the Public Interest (No. 201303034) and a grant from the Special Fund for Sichuan Science and Technology Support Program (No. 2013NZ0056).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethic approval The animal experiments were conducted in strict accordance with the recommendations in the China Regulations for the Administration of Affairs Concerning Experimental Animals (1988) and had been approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University (Approval Number BK2014-047), Sichuan, China.

References

Blackall PJ, Klaasen HLBM, Bosch HVD, Kuhnert P, Frey J (2002) Proposal of a new serovar of *Actinobacillus pleuropneumoniae*: serovar 15. Vet Microbiol 84:47–52

Bossé JT, Janson H, Sheehan BJ, Beddek AJ, Rycroft AN, Kroll JS, Langford PR (2002) *Actinobacillus pleuropneumoniae*: pathobiology and pathogenesis of infection. Microbes Infect 4:225–235

Bussière FI, Chaturvedi R, Cheng Y, Gobert AP, Asim M, Blumberg DR, Xu H, Kim PY, Hacker A, Jr CR (2005) Spermine causes loss of innate immune response to *Helicobacter pylori* by inhibition of inducible nitric-oxide synthase translation. J Biol Chem 280:2409

Chattopadhyay MK, Tabor CW, Tabor H (2003) Polyamines protect *Escherichia coli* cells from the toxic effect of oxygen. Proc Natl Acad Sci 100:2261–2265

Chaturvedi R, Asim M, Hoge S, Lewis ND, Singh K, Barry DP, De ST, Piazuelo MB, Sarvaria AR, Cheng Y (2010) Polyamines impair immunity to *Helicobacter pylori* by inhibiting L-arginine uptake required for nitric oxide production. Gastroenterology 139:1686

Chiang SM, Schellhorn HE (2012) Regulators of oxidative stress response genes in *Escherichia coli* and their functional conservation in bacteria. Arch Biochem Biophys 525:161–169

Chiers K, De Waele T, Pasmans F, Ducatelle R, Haesebrouck F (2010) Virulence factors of *Actinobacillus pleuropneumoniae* involved in colonization, persistence and induction of lesions in its porcine host. Vet Res 41:65

Childs AC, Mehta DJ, Gerner EW (2003) Polyamine-dependent gene expression. Cell Mol Life Sci 60:1394

Chung JW, Küster-Schöck E, Gibbs BF, Jacques M, Coulton JW (2012) Immunoproteomic analyses of outer membrane antigens of *Actinobacillus pleuropneumoniae* grown under iron-restricted conditions. Vet Microbiol 159:187–194

Durand JMB, Björk GR (2003) Putrescine or a combination of methionine and arginine restores virulence gene expression in a tRNA modification-deficient mutant of *Shigella flexneri*: a possible role in adaptation of virulence. Mol Microbiol 47:519–527

Fang X, Zhang Y, Gang L, Long Z, Liu S, Wang C (2013) The ClpP protease is required for the stress tolerance and biofilm formation in *Actinobacillus pleuropneumoniae*. PLoS ONE 8:e53600

Hara T, Matsuyama S, Tokuda H (2003) Mechanism underlying the inner membrane retention of *Escherichia coli* lipoproteins caused by Lol avoidance signals. J Biol Chem 278:40408–40414

Hillen S, Von BS, Köhler K, Reinacher M, Willems H, Reiner G (2014) Occurrence and severity of lung lesions in slaughter pigs vaccinated against *Mycoplasma hyopneumoniae* with different strategies. Prev Vet Med 113:580–588

Igarashi K, Kashiwagi K (2010) Modulation of cellular function by polyamines. Int J Biochem Cell Biol 42:39–51

Igarashi K, Ito K, Kashiwagi K (2001) Polyamine uptake systems in *Escherichia coli*. Res Microbiol 152:271

Iyer R, Delcour AH (1997) Complex inhibition of OmpF and OmpC bacterial porins by polyamines. J Biol Chem 272:18595–18601

Karatan E, Duncan TR, Watnick PI (2005) NspS, a predicted polyamine sensor, mediates activation of *Vibrio cholerae* biofilm formation by norspermidine. J Bacteriol 187:7434

Kashiwagi K, Hosokawa N, Furuchi T, Kobayashi H, Sasakawa C, Yoshikawa M, Igarashi K (1990) Isolation of polyamine transport-deficient mutants of *Escherichia coli* and cloning of the genes for polyamine transport proteins. J Biol Chem 265:20893–20897

Kashiwagi K, Miyamoto S, Suzuki F, Kobayashi H, Igarashi K (1992) Excretion of putrescine by the putrescine-ornithine antiporter encoded by the potE gene of *Escherichia coli*. Proc Natl Acad Sci USA 89:4529–4533

Kurihara S, Oda S, Kato K, Kim HG, Koyanagi T, Kumagai H, Suzuki H (2005) A novel putrescine utilization pathway involves gamma-glutamylated intermediates of *Escherichia coli* K-12. J Biol Chem 280:4602–4608

Kurihara S, Tsuboi YS, Kim HG, Kumagai H, Suzuki H (2009) The putrescine importer PuuP of *Escherichia coli* K-12. J Bacteriol 191:2776

Li Y, Cao S, Zhang L, Lau GW, Wen Y, Wu R, Zhao Q, Huang X, Yan Q, Huang Y, Wen X (2016) A TolC-like protein of *Actinobacillus pleuropneumoniae* is involved in antibiotic resistance and biofilm formation. Front Microbiol 7:1618

Liao Y, Deng J, Zhang A, Zhou M, Hu Y, Chen H, Jin M (2009) Immunoproteomic analysis of outer membrane proteins and extracellular proteins of *Actinobacillus pleuropneumoniae* JL03 serotype 3. BMC Microbiol 9:172

Miyamoto S, Kashiwagi K, Ito K, Watanabe S, Igarashi K (1993) Estimation of polyamine distribution and polyamine stimulation of protein synthesis in *Escherichia coli*. Arch Biochem Biophys 300:63–68

Nasrallah GK, Abdelhady H, Tompkins NP, Carson KR, Garduno RA (2014) Deletion of potD, encoding a putative spermidine-binding protein, results in a complex phenotype in *Legionella pneumophila*. Int J Med Microbiol 304:703–716

Patel CN, Wortham BW, Lines JL, Fetherston JD, Perry RD, Oliveira MA (2006) Polyamines are essential for the formation of plague biofilm. J Bacteriol 188:2355

Pistocchi R, Kashiwagi K, Miyamoto S, Nukui E, Sadakata Y, Kobayashi H, Igarashi K (1993) Characteristics of the operon for a putrescine transport system that maps at 19 minutes on the *Escherichia coli* chromosome. J Biol Chem 268:146–152

Rider JE, Hacker A, Mackintosh CA, Pegg AE, Woster PM, Jr CR (2007) Spermine and spermidine mediate protection against oxidative damage caused by hydrogen peroxide. Amino Acids 33:231–240

Schiller D, Kruse D, Kneifel H, Krämer R, Burkovski A (2000) Polyamine transport and role of potE in response to osmotic stress in *Escherichia coli*. J Bacteriol 182:6247–6249

Shah P, Swiatlo E (2008) A multifaceted role for polyamines in bacterial pathogens. Mol Microbiol 68:4–16

Shah P, Romero DG, Swiatlo E (2008) Role of polyamine transport in *Streptococcus pneumoniae* response to physiological stress and murine septicemia. Microb Pathog 45:167–172

Soksawatmaekhin W, Kuraishi A, Sakata K, Kashiwagi K, Igarashi K (2004) Excretion and uptake of cadaverine by CadB and its physiological functions in *Escherichia coli*. Mol Microbiol 51:1401–1412

Sugiyama S, Matsuo Y, Vassylyev DG, Matsushima M, Morikawa K, Maenaka K, Kashiwagi K, Igarashi K (1996a) The 1.8-Å X-ray structure of the *Escherichia coli* PotD protein complexed with spermidine and the mechanism of polyamine binding. Protein Sci 5:1984–1990

Sugiyama S, Vassylyev DG, Matsushima M, Kashiwagi K, Igarashi K, Morikawa K (1996b) Crystal structure of PotD, the primary receptor of the polyamine transport system in *Escherichia coli*. J Biol Chem 271:9519–9525

Tkachenko AG, Nesterova LY (2003) Polyamines as modulators of gene expression under oxidative stress in *Escherichia coli*. Biochemistry 68:850

Vega ALD, Delcour AH (1996) Polyamines decrease *Escherichia coli* outer membrane permeability. J Bacteriol 178:3715–3721

Yodsang P, Pothipongsa A, Mäenpää P, Incharoensakdi A (2014) Involvement of polyamine binding protein D (PotD) of *Synechocystis* sp. PCC 6803 in spermidine uptake and excretion. Curr Microbiol 69:417

Zhang F, Cao S, Zhu Z, Yang Y, Wen X, Chang YF, Huang X, Wu R, Wen Y, Yan Q (2016a) Immunoprotective efficacy of six in vivo-induced antigens against *Actinobacillus pleuropneumoniae* as potential vaccine candidates in murine model. Front Microbiol 38:728–732

Zhang L, Li Y, Wen Y, Lau GW, Huang X, Wu R, Yan Q, Huang Y, Zhao Q, Ma X, Wen X, Cao S (2016b) HtrA is important for stress resistance and virulence in *Haemophilus parasuis*. Infect Immun 84:2209–2219