

# A streptomycin resistance marker in *H. parasuis* based on site-directed mutations in *rpsL* gene to perform unmarked in-frame mutations and to verify natural transformation

Ke Dai<sup>1,\*</sup>, Xintian Wen<sup>1,\*</sup>, Yung-Fu Chang<sup>2,\*</sup>, Sanjie Cao<sup>1</sup>, Qin Zhao<sup>1</sup>, Xiaobo Huang<sup>1</sup>, Rui Wu<sup>1</sup>, Yong Huang<sup>1</sup>, Qigui Yan<sup>1</sup>, Xinfeng Han<sup>1</sup>, Xiaoping Ma<sup>1</sup> and Yiping Wen<sup>1</sup>

<sup>1</sup> College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, China

<sup>2</sup> Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, United States of America

\* These authors contributed equally to this work.

## ABSTRACT

*Haemophilus parasuis* is a member of the family *Pasteurellaceae* and a major causative agent of Glässer's disease. This bacterium is normally a benign swine commensal but may become a deadly pathogen upon penetration into multiple tissues, contributing to severe lesions in swine. We have established a successive natural transformation-based markerless mutation system in this species. However, the two-step mutation system requires screening of natural competent cells, and cannot delete genes which regulate natural competence per se. In this study, we successfully obtained streptomycin-resistant derivatives from *H. parasuis* wild type strain SC1401 by using ethyl methane sulfonate (EMS, CH<sub>3</sub>SO<sub>2</sub>OC<sub>2</sub>H<sub>5</sub>). Upon sequencing and site-directed mutations, we uncovered that the EMS-induced point mutation in *rpsL* at codon 43rd (AAA → AGA; K43R) or at 88th (AAA → AGA; K88R) confers a much higher streptomycin resistance than clinical isolates. We have applied the streptomycin resistance marker as a positive selection marker to perform homologous recombination through conjugation and successfully generated a double unmarked in-frame targeted mutant 1401D88Δ*tfoX*Δ*arcA*. Combined with a natural transformation-based knockout system and this genetic technique, multiple deletion mutants or attenuated strains of *H. parasuis* can be easily constructed. Moreover, the mutant genetic marker *rpsL* and streptomycin resistant phenotypes can serve as an effective tool to select naturally competent strains, and to verify natural transformation quantitatively.

**Subjects** Agricultural Science, Biotechnology, Microbiology, Veterinary Medicine

**Keywords** *rpsL*, Point mutation, EMS, *Haemophilus parasuis*, Streptomycin resistance, Natural transformation

## INTRODUCTION

*Haemophilus parasuis* (*H. parasuis*, HPS) is a Gram-negative, non-spore-forming, pleomorphic bacterium which is the etiological agent of Glässer's disease. NAD (V

Submitted 17 October 2017  
Accepted 19 December 2017  
Published 11 January 2018

Corresponding authors  
Xintian Wen, xintian3211@126.com  
Yiping Wen, yueliang5189@163.com

Academic editor  
Pedro Silva

Additional Information and  
Declarations can be found on  
page 16

DOI 10.7717/peerj.4253

© Copyright  
2018 Dai et al.

Distributed under  
Creative Commons CC-BY 4.0

OPEN ACCESS

factor), but not X factor, is necessary for the *in vitro* growth of *H. parasuis* in that the gene *nadV*, which encodes the nicotinamide phosphoribosyltransferase responsible for NAD salvage, is absent from the whole genome (Xu et al., 2011). The resulting illness is characterized by polyarthritis, fibrinous polyserositis and meningitis in pigs, producing significant mortality and morbidity in pig farms and leading to serious economic losses in the pork industry throughout the world (Oliveira & Pijoan, 2004). At least fifteen serotypes of varying virulence levels have been identified in this species (Kielstein & Rapp-Gabrielson, 1992). However, numerous gaps in our knowledge of molecular mechanisms of invading internal organs causing local and disseminated infection still remain at the present time (Zhou et al., 2016).

The conjugation process of creating markerless gene mutation is employed in an array of bacteria, which serves as a useful tool of constructing multi-gene scarless knock-outs. This facilitates in the construction of live attenuated vaccine strains and allows researchers to dissect the molecular mechanisms of virulence factors of *H. parasuis* and other bacterium (Heuermann & Haas, 1998; Oswald et al., 1999). A prerequisite for this process includes a marker-containing strain (Reyrat et al., 1998); a streptomycin resistance marker based on *rpsL* or *rrs* mutations is one of the most frequently used phenotypes (Kim et al., 2011).

Streptomycin (SM), belonging to aminoglycoside antibiotic, acts on the ribosome, inhibiting the translation of mRNA and therefore disrupting protein synthesis (Schatz, Bugie & Waksman, 2005). High-resolution melting and direct sequencing method in *Mycobacterium tuberculosis* and other species have been well established that streptomycin resistance mainly comes from mutations in the *rpsL* and *rrs* genes, which encode the ribosomal protein S12 and 16S rRNA, respectively (Wang et al., 2011). To generate SM resistant mutants by using spontaneous mutation, ultraviolet light (UV) induced mutations or transformation with plasmids containing streptomycin resistance markers has been used on various organisms (Timms et al., 1992; Skorupski & Taylor, 1996; Kim et al., 2011; Tsai et al., 2014). However, spontaneous or UV-induced mutants leading to a streptomycin resistance in *H. parasuis* probably occurs at a fairly low rate; SM resistant mutant generation in this organism was very inefficient in our hands using these strategies (data not shown). Moreover, the molecular mechanism of SM resistance is still unclear in this species.

EMS (ethyl methane sulfonate, methyl methanesulfonate) is a widely used chemical mutagen for inducing random mutations throughout the genome of plants (for example, *Cucumis sativus*) and some bacteria (for example, *Escherichia coli*) for its effective and efficient inductive effects (Parkhomchuk et al., 2009; Shah et al., 2015). Thus in this report, we introduced SM-resistance in *H. parasuis* based on the random somatic hypermutation effect of EMS and further performed site-directed point mutations in the same sites. We have previously demonstrated that *arcA* gene, encoding ArcA, a response regulator, contributes to the serum resistance and virulence in *H. parasuis* (Ding et al., 2016). ArcA and its sensor kinase ArcB, are one of the two-component signal transduction systems in *H. parasuis*. Since it is a global regulatory factor, whether it will affect natural transformation in *H. parasuis* has never been reported. *tfoX* gene is a core competence gene-regulon (by regulating cAMP-CRP complex to positively act on CRP-S sites in the promoter regions of late-competence genes). It has been well established that bacteria would lost its

natural transformability when *tfox*, whose homologue is *sxy* in *H. influenzae*, is deleted in *H. influenzae* and in many other bacteria (Sinha, Cameron & Redfield, 2009; Sinha, Mell & Redfield, 2012). However, the *tfox* gene cannot be deleted seamlessly by a previous technique established by us in that the successive markerless mutation system method requires a second round of natural transformation, whereas the  $\Delta tfox$  mutant has lost its transformability at the end of the first round (Zhang et al., 2015).

In the light of these findings, we used site-directed mutations in *rpsL* gene that confer a complete streptomycin resistance as a positive selection marker to successfully create a double gene mutant (1401D88 $\Delta tfox\Delta arcA$ ) in *H. parasuis* for further studying its biological functions. Moreover, we further used the genomic DNA of SM derivative to verify natural competence.

## MATERIAL AND METHODS

### Bacteria strains, plasmids and culture conditions

The bacteria strains, plasmids and primers used in this study are listed in Tables S1 and S2. Plasmids were propagated in *E. coli* DH5 $\alpha$  or S17-1 (pir) and bacteria were grown in liquid Lysogeny Broth (LB, Difco, USA) medium or on LB agar (Invitrogen, Shanghai, China) plate. When required, the medium was supplemented with kanamycin (Kan; 50  $\mu\text{g ml}^{-1}$ ) or ampicillin (Amp; 100  $\mu\text{g ml}^{-1}$ ) from Sigma-Aldrich, USA. *H. parasuis* was routinely cultured in Tryptic Soy Broth (TSB; Difco, Franklin Lakes, NJ, USA) or on Tryptic Soy agar (TSA; Difco, Franklin Lakes, NJ, USA) supplemented with 5% inactivated bovine serum (Solarbio, Beijing, China) and 0.1% (w/v) nicotinamide adenine dinucleotide (NAD; Sigma-Aldrich, St. Louis, MO, USA) (TSB++ and TSA++). Where necessary, the media were supplemented with streptomycin (SM; 25  $\mu\text{g ml}^{-1}$ ) or kanamycin (Kan; 50  $\mu\text{g ml}^{-1}$ ). Unless otherwise stated, all strains were grown at 37 °C.

### DNA manipulations

Bacterial genomic DNA was extracted using a TIANamp Bacteria DNA Kit (TIANGEN, Beijing, China). Small-scale plasmid DNA preparations were generated using a E.Z.N.A<sup>TM</sup> Plasmid Miniprep Kit (Omega Bio-Tek, Norcross, GA, USA). The DNA fragments were amplified in a C1000<sup>TM</sup> Thermal Cycler using the 2 $\times$ Taq Plus Master Mix (Vazyme, Jiangsu Sheng, China) or I-5<sup>TM</sup> 2 $\times$ High Fidelity Master Mix (MCLAB, South San Francisco, CA, USA). Purification of DNA fragments from the PCR reaction and the restriction digests were performed using the DNA Fragment Purification Kit Ver.4.0 (TaKaRa, Shiga, Japan). In-fusion segments were recombined to linearized pK18mobsacB (EcoRI/BamHI) using a ClonExpress II One Step Cloning Kit (Vazyme, Jiangsu Sheng, China). Restriction enzymes were purchased from TaKaRa. Point mutations were performed according to manual of *Fast* mutagenesis system Kit (TransGen Biotech, Beijing, China).

### Antibiotic and Ethyl Methane Sulfonate (EMS) susceptibility assays

Assays on the minimal inhibitory concentration were carried out as described by Li (Li et al., 2016b). Briefly, overnight-grown bacteria were grown in TSB++ with twofold serially

diluted SM (from 2  $\mu\text{g}/\text{mL}$  to 128  $\mu\text{g}/\text{mL}$ ; MIC-S) in a 96-well microtiter plate. The plates were incubated at 37 °C for 24 h, after which, the results were scored for growth or no growth. The experiments were independently performed at least three times in triplicate. The MIC of EMS (from 0.5 mM to 40 mM; MIC-E) was analyzed in sterilized tubes. The results were also scored for growth or no growth. Moreover, the MIC-S of EMS induced SC1401 derivatives were also determined using the above method.

The minimal bactericidal concentrations of EMS for *H. parasuis* (MBC-E) were determined using the method modified from Li and Zhang ([Zhang & Mah, 2008](#); [Li et al., 2016b](#)). Overnight-grown *H. parasuis* in sterilized tubes were exposed to different concentration of EMS (from 0.5 mM to 40 mM) for at least 12 h. Then, fresh TSB++ was used to replace the EMS medium and incubated for additional 16 h. The viability of bacteria was assessed by transferring a spot of the culture onto a TSA++ plate and incubation for 16 h. The experiments were independently performed at least three times in triplicate. Bacteria were serially passaged in TSB++ with MIC-E of EMS for five more generations for further determining the optimum inducing concentration of EMS.

In addition, the Oxford cup assay was used to evaluate the streptomycin resistance of wildtype *H. parasuis* SC1401, Hps32, Hps33 and derivatives 1401D88, 1401D43. Inocula were prepared and spread uniformly on TSA++ plates. One hundred microlitres of different concentrations (8,192  $\mu\text{g ml}^{-1}$  and 4,096  $\mu\text{g ml}^{-1}$  per well) of SM were added to the Oxford cups which were placed at equal distances above agar surfaces. The zone of inhibition for each concentration was measured after an extra 16 h-incubation at 37 °C. The experiments were independently performed at least three times in triplicate ([Shang et al., 2014](#)).

### **Ethyl Methane Sulfonate discontinuously inducing method**

Wild type SC1401 (whose genomic sequence has been deposited in GenBank under the accession number [NZ\\_CP015099.1](#)), which was SM sensitive and used as the parental strain, was cultured in TSB++ with an optimum concentration (10 mM) of EMS (AR, Biotopped, China) at 37 °C with agitation for 12 h. The culture (F1) was transferred into 5 mL of fresh TSB++ without EMS, in a “Healing Resurgence” step. The F2 was subcultured and induced by 10 mM of EMS again with the same method for F1, F2, F3, F4 generations. Each generation was plated onto TSA++ with appropriate SM antibiotic and incubated for 24 h. The visible single colonies were propagated and the DNA extracted for further identification.

### **Sequence analysis**

The 375-bp sequence of *rpsL* (locus tag: A4U84\_RS04600) were amplified from the visible single colonies' genomic DNA with primers *rpsL*-P1/P2 ([Table S2](#)). The PCR products were then purified. The products were cloned to pMD-19T(simple) for sequencing by BGI, Beijing, China. The RpsL protein sequences of *H. parasuis* were obtained by translating gene sequences using DNAMAN V6. Gene and protein sequences were then analyzed using BLAST in a comparison with that of wild type SC1401.

### Site-directed mutagenesis

To further delineate mutations in *rpsL*, rather than other random mutations in whole genome, principally contributing to SM resistance induced. The entire *rpsL* coding sequence and corresponding flanking regions (975 bp) were amplified from genomic DNA of wild type SC1401 using primers *rpsL*-PM-P1/P2 and subsequently cloned into suicide vector pK18mobSacB, generating wild type pkrpsL. Afterwards, *rpsL*-43-P1/P2 and *rpsL*-88-P1/P2 were used to amplify the whole pkrpsL backbone with respective point mutations at codon 43rd (A128G) and 88th (A263G) in ORF of *rpsL* with  $2 \times$  *Transtart Fastpfu* PCR SuperMix. Then the products were digested by DMT enzyme to move methylated template plasmids. Plasmids were subsequently mobilized into DMT competent cells by the CaCl<sub>2</sub> method. The mutants were confirmed by sequencing.

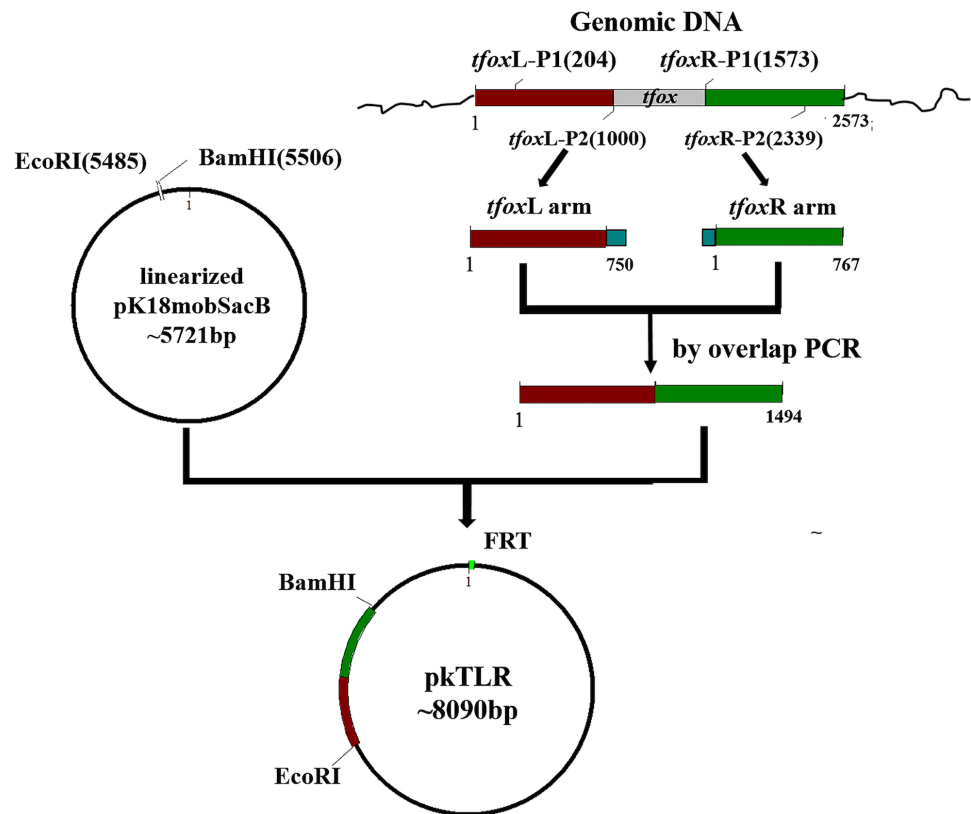
The wild type pkrpsL (control), mutant plasmids pkrpsL43 (A128G) and pkrpsL88 (A263G) were transformed into wild type strain SC1401 by electroporation. The electroporation parameters were: 1800 v/mm, 25  $\mu$ F, 200  $\Omega$ . The cells were screened by TSA++ containing 25  $\mu$ g ml<sup>-1</sup> of SM (although additional 10% sucrose together with antibiotic for screening transformants may be advantageous in order to prevent illegitimate recombinations, we found that once we obtained SM-resistant transformants, nearly all of them were expected derivatives after direct sequencing, even without the inclusion of 10% sucrose in the medium.). The visible single colonies were propagated and the DNA extracted and sequenced for further identification.

### Construction of plasmids pkTLR and pkALR

Plasmids pkTLR and pkALR were constructed for *tfox* and *arca* gene deletions and counter-selection in *H. parasuis* 1401D88, respectively. Here, we use pkTLR as an example to describe the process (Fig. 1). The 750-bp upstream homologous arm region and the 767-bp down homologous arm region of *tfox* were amplified using primers *tfox*L-P1/P2 and *tfox*R-P1/P2 from the genome of SC1401 (or 1401D88), respectively. These two flanking fragments were then purified using Qiaquick spin column (Qiagen, Hilden, Germany) and integrated by overlap extension PCR with *tfox*L-P1/*tfox*R-P2, after which the in-fusion segment was recombined to linearized pK18mobsacB (EcoRI/BamHI) using ClonExpress II. One Step Cloning Kit (Vazyme, Jiangsu Sheng, China). After confirmation by PCR and sequencing, the resulting plasmid, pkTLR, was mobilized into S17-1 ( $\lambda$ pir) by the CaCl<sub>2</sub> method.

### Construction of unmarked in-frame targeted mutant 1401D88 $\Delta$ *tfox* $\Delta$ *arca* of *H. parasuis* 1401D88

An unmarked *tfox* mutant was constructed using a two-step selective method (Fig. 2; Fig. 3). DNA was mobilized from *E. coli* S17-1 ( $\lambda$ pir) to *H. parasuis* using a filter mating technique adapted by Oswald with some modification (Oswald *et al.*, 1999). Briefly, S17-1 ( $\lambda$ pir) harboring pkTLR and 1401D88 strains were grown to exponential phase in SOC and TSB++, respectively. The bacteria cultures were centrifuged at  $4,350 \times g$  for 5 min; the pellet was resuspended in 1 mL of TNM-buffer (1 mM Tris-HCl pH 7.2, 10 mM MgSO<sub>4</sub>, 100 mM NaCl). Aliquots corresponding to 0.5 ml of donor and 1 ml of recipient (each adjusted to

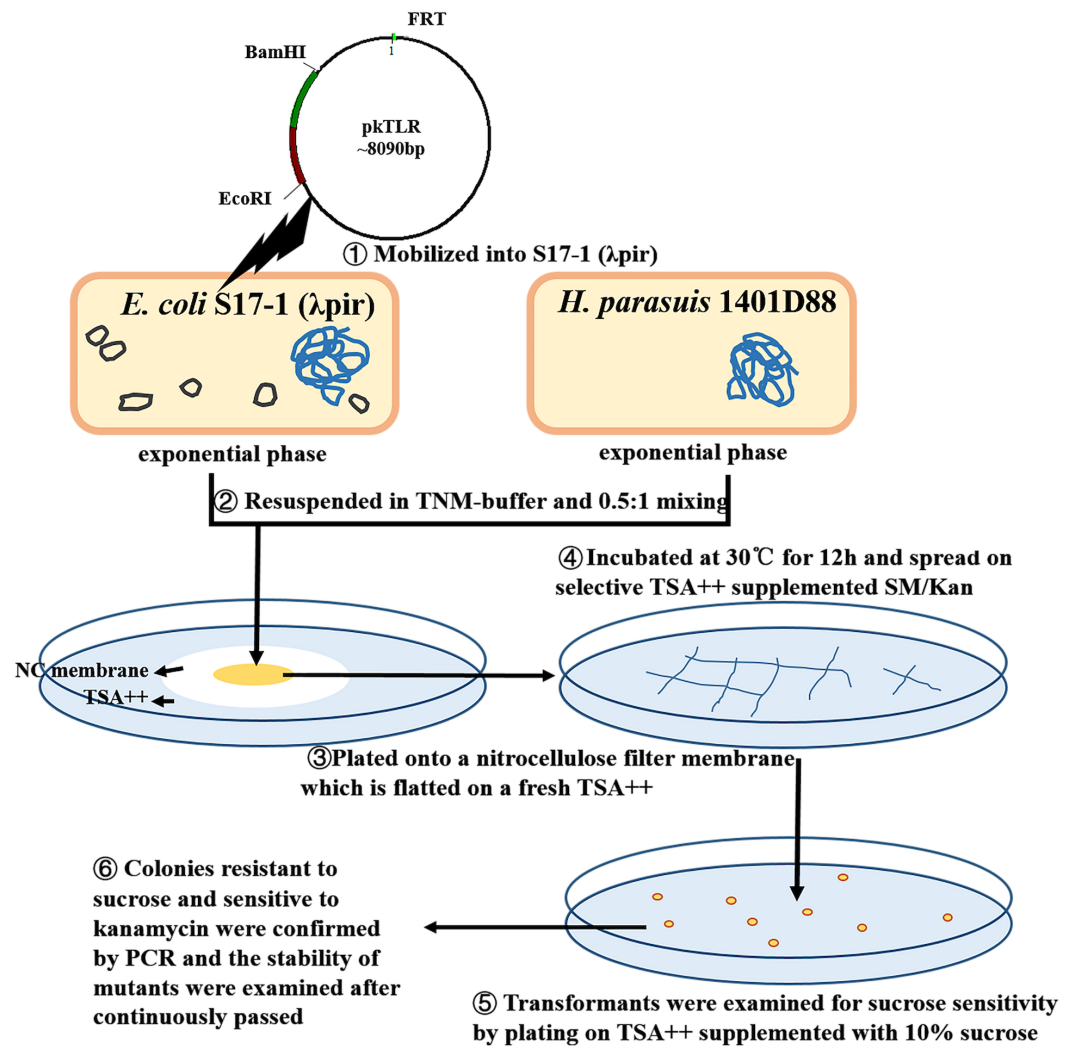


**Figure 1** Scheme for the construction of *pkTLR*. Primer pairs *tfoxL*-P1/P2 and *tfoxR*-P1/P2 were used to amplify the upstream and downstream arm of *tfoX* gene, respectively. Each with 21 bp homologous fragment for recombination to each other and to vector *pK18mobSacB*. Both products were integrated by overlap extension PCR with primers *tfoxL*-P1/*tfoxR*-P2. The resulting segment was then cloned into *pK18mobSacB* by a ClonExpress II One Step Cloning Kit (Vazyme, Jiangsu Sheng, China).

Full-size [DOI: 10.7717/peerj.4253/fig-1](https://doi.org/10.7717/peerj.4253/fig-1)

an  $OD_{600} = 1$ ) were mixed and plated onto a nitrocellulose filter membrane (0.45  $\mu\text{m}$  pore size, 2.5 cm diameter; Millipore, Darmstadt, Germany). The NC membrane with bacterial mixture was placed onto TSA++ plates and incubated at 30 °C for 12 h. Eventually, the cultures were scraped up and spread on selective TSA++ supplemented with 25  $\mu\text{g ml}^{-1}$  of SM and 50  $\mu\text{g ml}^{-1}$  of Kan and incubated at 37 °C for 24 h. The transformants were then examined for sucrose sensitivity by plating on TSA++ supplemented with 10% sucrose (UP; Amresco, Solon, OH, USA) and 25  $\mu\text{g ml}^{-1}$  of SM. PCR was carried out to confirm the appropriate deletion in colonies resistant to sucrose and sensitive to kanamycin. Afterwards, the mutants were continuously passed to 20 times on TSA++ and primers *tfoX*-P1/P2, *tfoxL*-P1/*tfoxR*-P2 and *Hps*-P1/P2 were adopted for further confirming stability of the desired mutant.

The effectiveness of our novel protocol for generating a double-gene mutant was confirmed when we further deleted the *arcA* gene from 1401D88 $\Delta$ *tfoX*. Likewise, the process was repeated as above, using a different primer set. The upstream and downstream

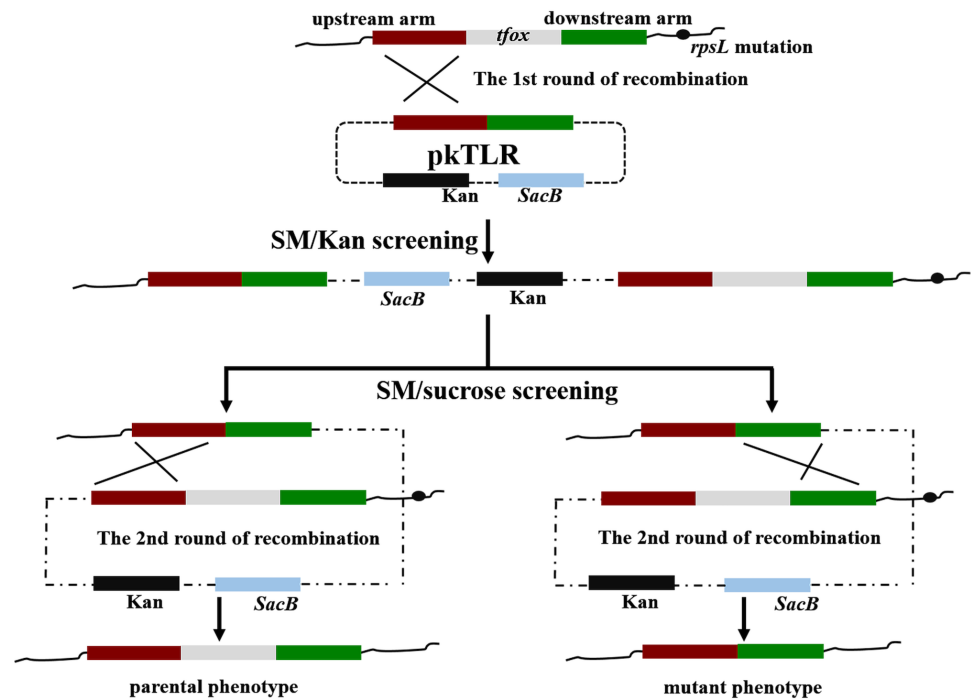


**Figure 2** Protocols of constructing in-frame mutants in *H. parasuis* using conjugation method.

Full-size [DOI: 10.7717/peerj.4253/fig-2](https://doi.org/10.7717/peerj.4253/fig-2)

homologous arms regions of *arcA* were amplified using primers *arcAL*-P1/P2 and *arcAR*-P1/P2, respectively. Primers *arcA*-P1/P2, *arcAL*-P1/*arcAR*-P2 and Hps-P1/P2 were adopted for further confirming the stability of the desired mutant.

The whole extract of 1401D88 $\Delta$ *fox* $\Delta$ *arcA* was further analyzed by western blotting. Briefly, the whole-cell lysates of the 1401D88, 1401D88 $\Delta$ *fox* $\Delta$ *arcA* mutant strain and SC1401 strain were analyzed by 12% SDS-PAGE electrotransferred to a polyvinylidene fluoride membrane (PVDF membrane). After being activated in methanol for 30 s and blocked with 5% BSA in TBST at room temperature (RT) for 30 min to 1 h, the membrane was incubated at RT for 1 h or overnight with mouse antiserum against recombinant Tfox or ArcA as the primary antibody, respectively. The membrane was rinsed 3–5 times with no less than 25 mL of TBST between incubations. Horseradish peroxidase (HRP)-conjugated rabbit-anti-mouse IgG (Earth, Hong Kong) were used as the secondary antibody (1:10,000



**Figure 3** Principles of obtaining gene mutant phenotype (in-frame mutation) by homologous recombination. The whole pKTLR recombines to the *tfox* loci on the chromosome by the first allelic replacement thus the transformants can be selected by TSA++ supplemented with SM/Kan; through a second allelic replacement, we obtained mutants through PCR screening.

Full-size DOI: [10.7717/peerj.4253/fig-3](https://doi.org/10.7717/peerj.4253/fig-3)

dil.). The membrane was finally developed with Immun-Star Western C Kit (Biorad, USA) according to the manufacturer's instructions (Ding *et al.*, 2016).

### Using genomic DNA of 1401D88 and 1401D43 to verify natural transformation

The genomic DNAs of *H. parasuis* SC1401 derivatives 1401D88 and 1401D43 via site-directed point mutations in *rpsL* gene (as indicated in 'DNA manipulations') that confer SM resistance to *H. parasuis* were used to verify natural transformation capacity of *H. parasuis* strain SC1401; genomic DNA of SC1401 $\Delta$ *htrA*, HPS32, HPS33 and the plasmid DNA pKBHK were used as controls. The protocol for natural transformation was performed as previously described with some modifications (Bigas *et al.*, 2005). Briefly, recipient bacteria were grown in TSB++ to log phase or to an  $OD_{600} = 1.8$  (about  $2.8 \times 10^9$  cfu/mL). The bacteria were then spotted on TSA++, cultured overnight at 37 °C and resuspended in TSB++ at  $20 \times 10^{10}$  cfu/mL. A 20  $\mu$ L aliquot of suspension was supplemented with 1  $\mu$ g of donor DNA. The mixture was incubated for 10 min at 37 °C and then spread in a small area on TSA++. Cultures were incubated for an additional five hours at 37 °C to induce expression of antibiotic resistance. Afterwards, bacteria were harvested and resuspended and plated on TSA++ with SM in concentrations ranging from 25  $\mu$ g ml<sup>-1</sup> to no antibiotic. Bacteria were incubated for 36 h.



**Table 1** Susceptibility of *H. parasuis* strains to streptomycin (SM) and ethyl methane sulfonate (EMS).

Object	Test index <sup>a</sup>	Strains <sup>b</sup>				
		SC1401	1401D43	1401D88	HPS32	HPS33
SM	MIC-S ( $\mu\text{g/mL}$ )	16	> 4,096	> 4,096	16	16
	MBC-S ( $\mu\text{g/mL}$ )	24	> 4,096	> 4,096	28	32
EMS	MIC-E (mM)	20	–	–	–	–
	MBC-E (mM)	30	–	–	–	–

**Notes.**

<sup>a</sup>MIC-S/E, minimal inhibitory concentration of SM/EMS; MBC-S/E, minimal bactericidal concentrations of SM/EMS.

<sup>b</sup>1401D43 and 1401D88 are completely resistant to SM.

After incubation, the colonies on TSA++ and on selective plates were counted. Transformation frequencies were determined from the number of antibiotic-resistant CFU  $\text{mL}^{-1}$  divided by the total CFU  $\text{mL}^{-1}$  scored on nonselective agar. Transformation efficiency was evaluated by calculating number of transformed CFU per  $\mu\text{g}$  of donor DNA.

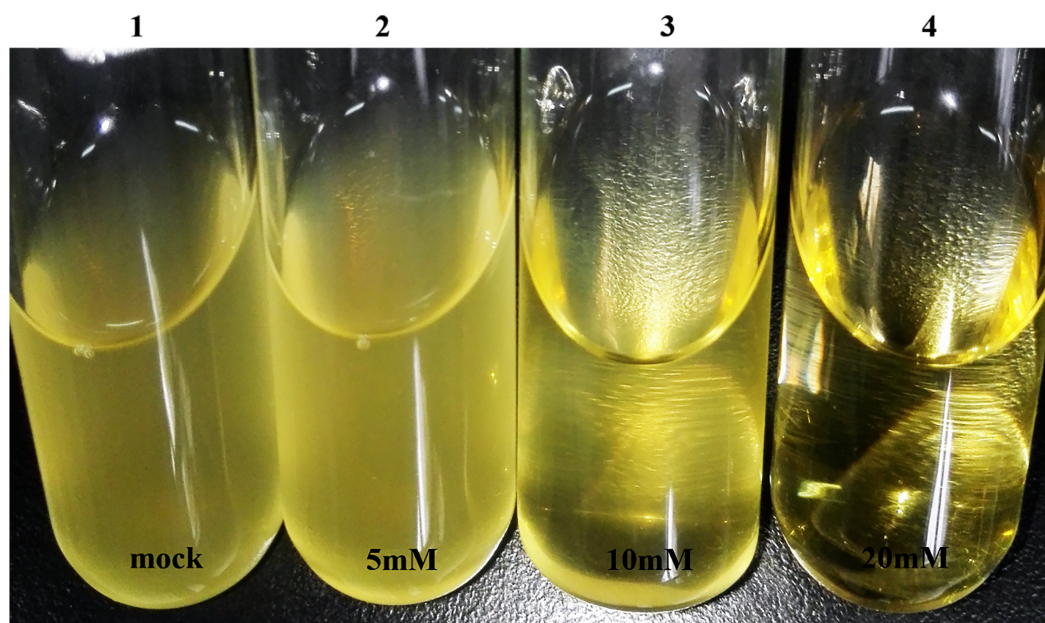
**Statistical analysis**

The statistical analysis was performed using R studio (loaded with R V3.3.2) for Windows (*RStudio Team, 2015*). Comparison of several test series was evaluated by analysis of variance (ANOVA). The significance of differences between groups was calculated using Student's *t*-test. A *P* value < 0.05 was considered to be statistically significant (\*), and <0.01 highly significant (\*\*).

**RESULTS****Determination of MIC & MBC of streptomycin and ethyl methane sulfonate in *H. parasuis***

To investigate an appropriate inhibiting concentration of SM and EMS, we determined minimal inhibitory concentration of SM (MIC-S) and EMS (MIC-E) for *H. parasuis* SC1401. The results showed that MIC-S for SC1401 to be  $16 \mu\text{g ml}^{-1}$ ; however,  $24 \mu\text{g ml}^{-1}$  of SM was bactericidal (Table 1). Hence  $25 \mu\text{g ml}^{-1}$  of SM was employed in later experiments to screen out SM-resistant derivatives from wild type SC1401. By comparison, EMS was shown to have fairly strong antibacterial activity. As shown in Fig. 4, we could see flocculent bacteria when the culture was exposed to 10 mM of EMS, but not in the TSB++ with 20 mM of EMS. Therefore, twenty millimoles per liter of EMS was determined to be the MIC-E of *H. parasuis* SC1401 (Table 1 and Fig. 4).

To determine optimal inducing, subinhibitory concentrations of EMS, we tested the minimal bactericidal concentrations (MBC-E). Overnight-grown seed broth was subcultured in fresh TSB++ with different concentration of EMS (from 0.5 mM to 40 mM) and subcultured again with TSB++ with no EMS. The results showed that 30 mM of EMS was the definitive MBC-E (Table 1).



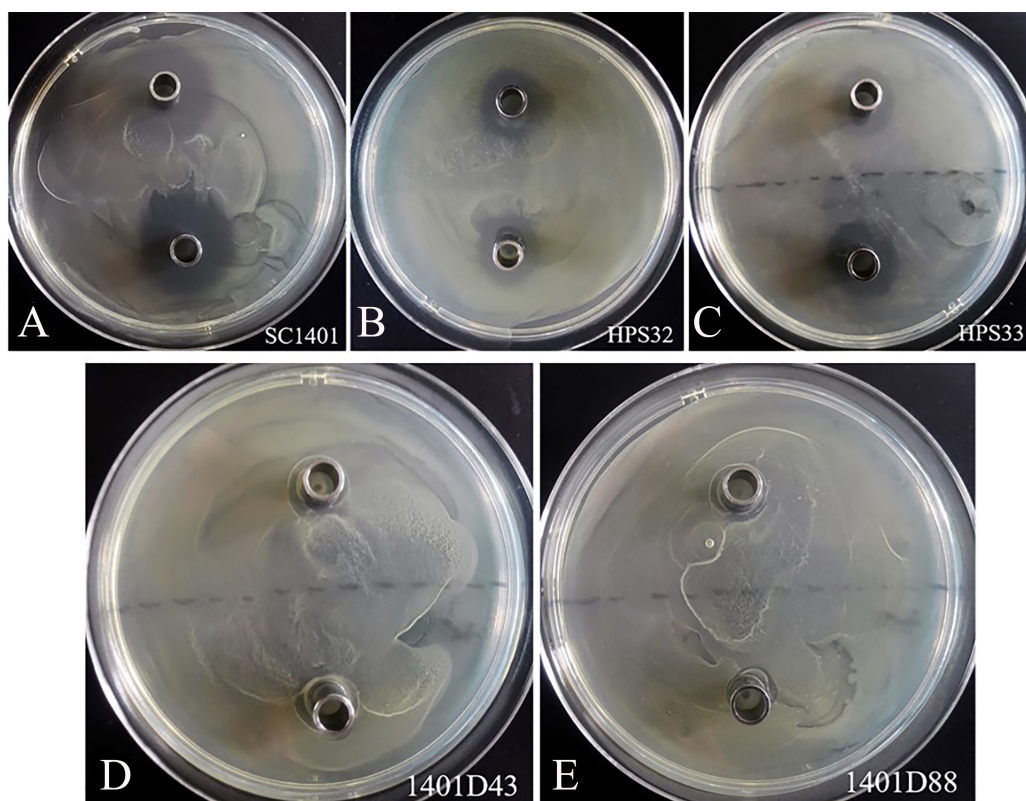
**Figure 4** High concentration of EMS exhibits obvious bacteriostatic activity. Visible flocculent bacteria under 10 millimoles per liter of EMS were more easily subjected to discontinuous induction; these were serially passaged.

Full-size DOI: 10.7717/peerj.4253/fig-4

### The EMS-induced derivatives of SC1401 and MIC-S assay

As shown in Table 1, thirty milli-moles per liter of EMS has strong bactericidal activity (this concentration was determined to be the MBC-E), whereas 10 mM of EMS was shown to be the MIC-E (Fig. 4 and Fig. S1). Therefore, in consideration of maximizing the inductive effect of EMS, we used a discontinuous induction method in our experiments. The culture in a 10 mM of EMS-containing TSB++ was transferred into 5 mL of fresh TSB++ without any EMS, in a “Healing Resurgence” step of our protocol. Each generation was plated onto TSA++ with appropriate SM antibiotic and incubated for 24 h. In this way, we obtained SM-resistant generations in F7, F9 and F11. Among them, mutations in *rpsL* at codon 43rd (A128G) and 88th (A263G) were confirmed by sequencing.

By comparison, these two kinds of mutations conferred complete resistance to SM (Table 1). The two derivatives can survive well at a concentration of at least  $4,096 \mu\text{g ml}^{-1}$  of SM. As a control, *H. parasuis* wild type strains SC1401, HPS32, HPS33 can only survive in less than  $16 \mu\text{g ml}^{-1}$  of SM/TSB++. We also performed the Oxford cup assay to visually demonstrate different levels of SM-resistance. As shown in Fig. 5, 1401D43 and 1401D88 can form complete bacteria lawns on TSA++ with both  $100 \mu\text{l}$  of  $8,192 \mu\text{g ml}^{-1}$  and  $4,096 \mu\text{g ml}^{-1}$  of SM per well, while inhibition zones can be easily recognized for wild type *H. parasuis*, indicating the successfully artificially EMS-induced products were far more resistant to SM than wild type SC1401.



**Figure 5** Streptomycin resistance analysis based on Oxford cup assay. Oxford cups containing  $8,192 \mu\text{g ml}^{-1}$  and  $4,096 \mu\text{g ml}^{-1}$  per well of SM were used to analysis bacterial resistance. It demonstrated that (D, E) the derivatives 1401D43 and 1401D88 were completely resistant to SM, whereas (A–C) wild type *H. parasuis* exhibited distinct bacterial inhibition zones.

Full-size DOI: 10.7717/peerj.4253/fig-5

### *rpsL* hot mutations

It has been well established in procaryotic organisms that streptomycin hinders the functioning of ribosome by binding to 16S rRNA helices and ribosomal protein S12, encoded by *rrs* and *rpsL*, respectively. SM also interacts with decoding site and shifts 16S rRNA helix in the direction of ribosomal protein S12 which in turn induces miscoding by stabilising the closed confirmation of 30S ribosomal subunit (Suriyanarayanan *et al.*, 2016). We directly sequenced the *rpsL* and *rrs* genes for wild-type strains Hps32, Hps32 and artificially induced SM-resistant strains. We used BLAST and these reads to query the *rpsL* and *rrs* sequences of *H. parasuis* SC1401. The results of DNA sequencing showed that all these SM-resistant strains harbored *rpsL* mutations. Comparatively, point mutations at codon 88th (AAA → AGA; K88R) and at codon 43rd (AAA → AGA; K43R) confer much higher SM-resistance on the EMS-induced derivatives than *H. parasuis* wild type strains Hps32, Hps32, in which random mutations of synonymous codon have been introduced (Fig. 6), indicating that the mutations at codon 43rd and 88th are the core regions blocking SM binding in *H. parasuis*, whilst other point mutations occurred less frequently. The mostly highly-resistant SM mutations occur in these two codons.

Wild type SC1401	ATGGCAACTATCAACCGACTACTAGCAAAACCGCGTGTGAAAAGGTTGTA AAAAGCAACGCTTCCCTCGCTTTGAGGCTTGCCCGCAGAAACGGTGTGT	100
SC1401 derivative-1401D88	ATGGCAACTATCAACCGACTACTAGCAAAACCGCGTGTGAAAAGGTTGTA AAAAGCAACGCTTCCCTCGCTTTGAGGCTTGCCCGCAGAAACGGTGTGT	100
SC1401 derivative-1401D43	ATGGCAACTATCAACCGACTACTAGCAAAACCGCGTGTGAAAAGGTTGTA AAAAGCAACGCTTCCCTCGCTTTGAGGCTTGCCCGCAGAAACGGTGTGT	100
Wild type HPS32	ATGGCAACTATCAACCGACTACTAGCAAAACCGCGTGTGAAAAGGTTGTA AAAAGCAACGCTTCCCTCGCTTTGAGGCTTGCCCGCAGAAACGGTGTGT	100
Wild type HPS33	ATGGCAACTATCAACCGACTACTAGCAAAACCGCGTGTGAAAAGGTTGTA AAAAGCAACGCTTCCCTCGCTTTGAGGCTTGCCCGCAGAAACGGTGTGT	100
Consensus	atggcaactatacaacccgactagtacgcaaaacccgctgtgaaaaaggttgtaaaaagcaacgcttccctcgctttgaggcttgcccgagaaacgggtgtgt	
	<b>core region of binding SM</b>	<b>synonymous codons</b>
Wild type SC1401	GTACTCGTGTATACACTACAACCTCTTAAAPCCAACTCAGCGTTACGTAAGTATCTCGTATCCGCTTAACAPACGGTTTTGAAGTAACTTCTTATAT	200
SC1401 derivative-1401D88	GTACTCGTGTATACACTACAACCTCTTAAAPCCAACTCAGCGTTACGTAAGTATCTCGTATCCGCTTAACAPACGGTTTTGAAGTAACTTCTTATAT	200
SC1401 derivative-1401D43	GTACTCGTGTATACACTACAACCTCTTAAAPCCAACTCAGCGTTACGTAAGTATCTCGTATCCGCTTAACAPACGGTTTTGAAGTAACTTCTTATAT	200
Wild type HPS32	GTACTCGTGTATACACTACAACCTCTTAAAPCCAACTCAGCGTTACGTAAGTATCTCGTATCCGCTTAACAPACGGTTTTGAAGTAACTTCTTATAT	200
Wild type HPS33	GTACTCGTGTATACACTACAACCTCTTAAAPCCAACTCAGCGTTACGTAAGTATCTCGTATCCGCTTAACAPACGGTTTTGAAGTAACTTCTTATAT	200
Consensus	gtactcgtgtatatacactacaactccta <sup>aa</sup> aacc aactcagcgttacgtaagtagtgcgtatccgcttaacaaacggTTTTGAAGTAACTTCTTATAT	
	<b>K43R synonymous codons</b>	<b>core region of binding SM</b>
Wild type SC1401	CGGIGGTGAAGTCAAAACCTTCAAGAACACAGTGTGTATTAATCCGGTGGTGGTGGTTAAGACTTACCAGGTGTACGTTATCACACTGIACGTGGT	300
SC1401 derivative-1401D88	CGGIGGTGAAGTCAAAACCTTCAAGAACACAGTGTGTATTAATCCGGTGGTGGTGGTTAAGACTTACCAGGTGTACGTTATCACACTGIACGTGGT	300
SC1401 derivative-1401D43	CGGIGGTGAAGTCAAAACCTTCAAGAACACAGTGTGTATTAATCCGGTGGTGGTGGTTAAGACTTACCAGGTGTACGTTATCACACTGIACGTGGT	300
Wild type HPS32	CGGIGGTGAAGTCAAAACCTTCAAGAACACAGTGTGTATTAATCCGGTGGTGGTGGTTAAGACTTACCAGGTGTACGTTATCACACTGIACGTGGT	300
Wild type HPS33	CGGIGGTGAAGTCAAAACCTTCAAGAACACAGTGTGTATTAATCCGGTGGTGGTGGTTAAGACTTACCAGGTGTACGTTATCACACTGIACGTGGT	300
Consensus	cggtggtgaaggtcaaaaccttcaagaacacagtggtgtat <sup>aa</sup> tccgggtgggtgggtggtta <sup>aa</sup> gacttaccaggtgtacgtttatcacactgtacgtggt	
	<b>synonymous codons</b>	<b>K88R</b>
Wild type SC1401	GCACCTTGACTGTCCAGGCGTTAAAGACCGTAAACAGGTCGTTCTAAATACGGCGTTAAACGTCCTAAGTCTTA	374
SC1401 derivative-1401D88	GCACCTTGACTGTCCAGGCGTTAAAGACCGTAAACAGGTCGTTCTAAATACGGCGTTAAACGTCCTAAGTCTTA	374
SC1401 derivative-1401D43	GCACCTTGACTGTCCAGGCGTTAAAGACCGTAAACAGGTCGTTCTAAATACGGCGTTAAACGTCCTAAGTCTTA	374
Wild type HPS32	GCACCTTGACTGTCCAGGCGTTAAAGACCGTAAACAGGTCGTTCTAAATACGGCGTTAAACGTCCTAAGTCTTA	374
Wild type HPS33	GCACCTTGACTGTCCAGGCGTTAAAGACCGTAAACAGGTCGTTCTAAATACGGCGTTAAACGTCCTAAGTCTTA	374
Consensus	gcacttgactgtccaggcgttaaagaccgtaaacaggctcgttctaataacggcgttaaaccgtcctaagctcta	

**Figure 6** Mutation sites in *rpsL* gene of derivatives 1401D43 and 1401D88 as well as two wild type SM-resistant *H. parasuis*. The hot mutation sites in *H. parasuis* indicates that the K→R mutations in 43rd and 88th codons in RpsL conferred resistance to SM. While we could only find synonymous mutations in wild type HPS32 and HPS33 within *rpsL* gene, so their SM-resistance may come from the *rrs* mutations (have been identified but not shown) or other genetic factors. However, these kinds of mutations or factors contribute little in conferring SM-resistance.

Full-size  DOI: 10.7717/peerj.4253/fig-6

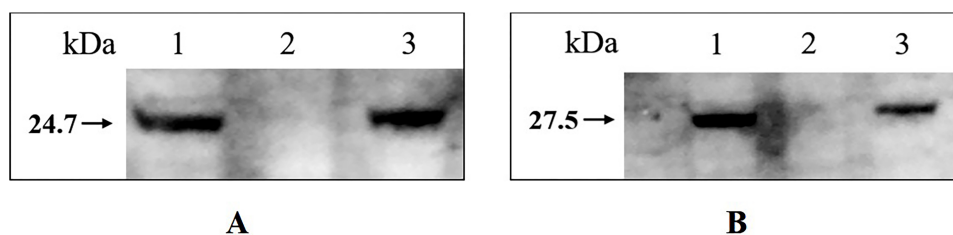
However, we haven't found a derivative with simultaneous mutations in these two sites. By comparison, *rrs* mutation could be found in derivative 1401D43 (in which only G920A have been identified), HPS32 and HPS33 (in which almost fifteen point-mutations have been identified, data not shown), but obviously not a greater factor of inducing the high resistance to SM (Table 1 and Fig. 5). We didn't find any mutations in *rrs* in 1401D88.

### Identification of successful construction of pkTLR and pkALR

Further investigation of Kan-resistant colonies showed that the in-fusion segments of up/downstream regions of *tfox* was successfully integrated into linearized pK18mobsacB. PCR runs with primers *tfoxL*-P1/*tfoxL*-P2, *tfoxR*-P1/*tfoxR*-P2 and *tfoxL*-P1/*tfoxR*-P2 demonstrated that the products were amplified as expected, which was followed by sequencing for further confirmation (Fig. S2). The plasmid pkTLR was then used to create unmarked in-frame *tfox* mutations in *H. parasuis*. Likewise, the plasmid pkALR was generated and identified as above. PCR runs were carried out using primers *arcAL*-P1/*arcAL*-P2, *arcAR*-P1/*arcAR*-P2 and *arcAL*-P1/*arcAR*-P2.

### Construction of an unmarked mutation of *tfox* and *arcA* genes in *H. parasuis* 1401D88

Based on a two-step natural transformation mutagenesis method, we used a 'dual' process with one round of SM selection and a subsequent sucrose counterselection. The in-fusion segment was successfully transferred to the chromosomal DNA of recipient strain and subsequently integrated into the host *tfox* loci through homologous recombination, yielding the *in situ* deletion. Here, the method of EMS induced high level-resistance to SM plays an important role in weeding out donor strain S17-1 ( $\lambda$ pir), thus allowing us to generate unmarked mutants in *H. parasuis* that are multi-drug susceptible.



**Figure 7** Western blotting analysis of wild type *H. parasuis* and mutant. The whole-cell lysates of the 1401D88, mutant and SC1401 strains were detected using *tfoX* and *arcA* mouse antiserum, respectively. The derivatives 1401D88 displayed a similar band to the parent strain SC1401, while 1401D88 $\Delta$ *tfoX* $\Delta$ *arcA* strain did not. (A) Western blotting analysis using *tfoX* mouse antiserum as primary antibody. Lane 1, 1401D88 strain; Lane 2, 1401D88 $\Delta$ *tfoX* $\Delta$ *arcA* strain; Lane 3, SC1401 strain. (B) Western blotting analysis using *arcA* mouse antiserum as primary antibody. Lane 1, 1401D88 strain; Lane 2, 1401D88 $\Delta$ *tfoX* $\Delta$ *arcA* strain; Lane 3, SC1401 strain.

Full-size DOI: 10.7717/peerj.4253/fig-7

The plasmid pKTLR was integrated into the two flanking regions of *tfoX* gene at the genomic site, so that the original *tfoX* gene was replaced seamlessly. SM and Kan positive selection was highly successful, and the PCR tests validated both the insertion of the kan cassette and *SacB* gene in most of the Kan-resistant clones (Fig. S3A). In the second round of homologous recombination, the backbone of pK18mobSacB was removed, followed by sucrose counterselection and kanamycin susceptibility testing. PCRs were carried out to verify the appropriate deletion in colonies resistant to 10% sucrose and 25  $\mu\text{g ml}^{-1}$  of SM but sensitive to Kan (Fig. S3B).

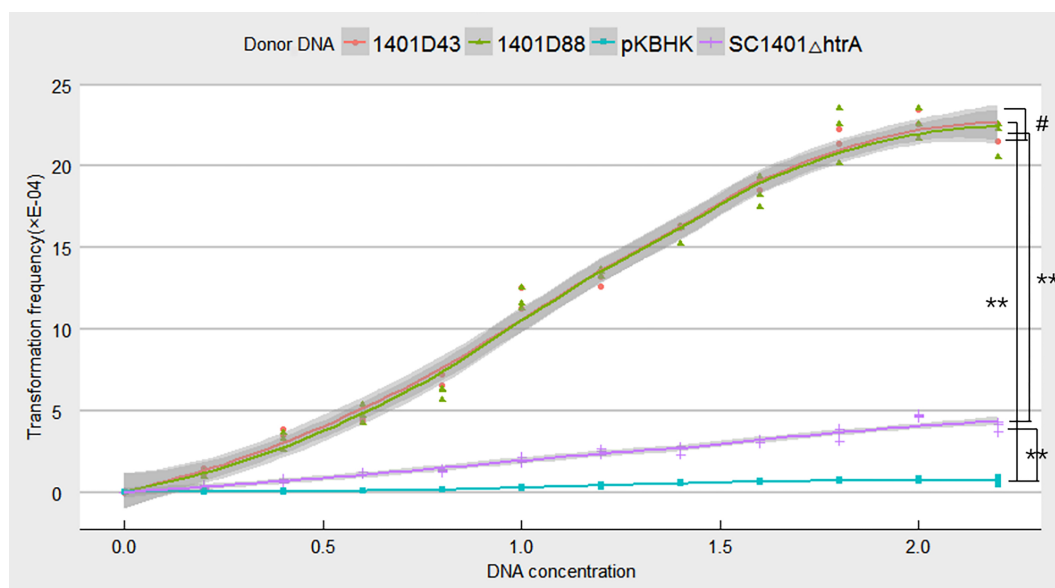
The second round is to remove *arcA* gene from 1401D88 $\Delta$ *tfoX*. Likewise, pKALR was used to create in-frame *arcA* knock-out mutant. PCR runs were carried out to confirm the appropriate deletion in transformants resistant to sucrose and 25  $\mu\text{g ml}^{-1}$  of SM but sensitive to 20  $\mu\text{g/ml}$  Kan.

### Confirmation of 1401D88 $\Delta$ *tfoX* $\Delta$ *arcA* by western blotting

Western blotting confirmed that *tfoX* and *arcA* can be detected in the whole-cell extract of wild type *H. parasuis* SC1401 and 1401D88, but not in 1401D88 $\Delta$ *tfoX* $\Delta$ *arcA* (Fig. 7). This result further confirms the successful deletion of *tfoX* and *arcA* genes in strain 1401D88.

### Verification of natural competence

Point mutations in *rpsL* which don't confer negative effect on growth were assumed to be more effective in verifying natural competence (see Fig. S4 for comparison of growth rates of *H. parasuis* mutants and wild type SC1401). To confirm this hypothesis, we tested the natural frequencies of different donor DNA, including genomic DNA of *H. parasuis* 1401D43, 1401D88, SC1401 $\Delta$ *htrA* (in which the Kan cassette had been stably inserted.) and the plasmid DNA pKBHK which was previously used to create *htrA* knock-out mutant in *H. parasuis* and to screen natural competent cells, as well as the naturally SM-resistant strains HPS32 and HPS33. The results showed that transformation frequencies of 1401D43, 1401D88, SC1401 $\Delta$ *htrA* genomic DNA after an extended growth period (13 h) on TSA++ were considerably higher than those for pKBHK construct. Genomic DNA of HPS32 and HPS32 couldn't confer SM-resistance to SC1401, which also in support of the elucidation



**Figure 8** Natural transformation frequency of different donor DNA. Lines are achieved by mean values of three replicates fitting, and shaded areas indicate the confidence interval between three replicates.

Full-size DOI: [10.7717/peerj.4253/fig-8](https://doi.org/10.7717/peerj.4253/fig-8)

highlighted in the “Introduction” that spontaneous mutations in this species occur at a fairly low level. The transformation frequencies of derivative genomic DNA reached a fairly high level (about  $2.458 \times 10^{-3}$ ) compared to SC1401Δ*htrA* genomic DNA. However, the highest transformation frequency of genomic DNA of SC1401Δ*htrA* reached to  $4.748 \times 10^{-4}$  (Fig. 8). Furthermore, genomic DNA of 1401D43, 1401D88 can be actively taken up by recipient cells. The transformation frequencies of 1401D43, 1401D88 DNA derived from point mutations in SC1401 are significantly higher than SC1401Δ*htrA* genomic DNA which carries a Kan<sup>R</sup> cassette in the *htrA* loci. In independent repeated experiments, more than thirty transformants were directly sequenced for further identification of desired mutations in *rpsL*.

## DISCUSSION

*H. parasuis* is one of the most economically significant swine pathogens at present in the swine industry throughout the world. This pathogen usually invades barrier of blood vessels and causes multiple syndromes when the host’s immunity is suppressed. At least 40 *H. parasuis* species have been sequenced and large array of potential virulence factors have been found (Li et al., 2016a); however, their pathogenic mechanisms and evolution remain largely unknown. Previous studies on gene deletion usually based on natural transformation system of highly transformable strains, such as SC1401 (Zhang et al., 2016), SC096 (Zhang et al., 2014a), EP3 (Ding et al., 2016), CF7066 (Huang et al., 2016). However, the natural transformation technology relies heavily on screening of natural competent cells, which is inefficient since not all bacteria in *H. parasuis* are competent cells, at least under the existent experiment cues and conditions (Seitz & Blokesch, 2013; Johnston et al., 2014).

Although EMS may introduce hypermutations in *H. parasuis*, certain concentration of EMS (more than 30 mM) exhibits a bactericidal effect, thus we tested the MIC-E and MBC-E and subsequently found 10 mM of EMS is an optimized concentration for inducing *rpsL* mutations *H. parasuis*. We couldn't obtain SM-resistant derivatives in the earlier attempts using EMS. A discontinuous induction method in which one alternate step labelled "Healing Resurgence" was applied. With this technique, we successfully obtained an array of SM-resistant derivatives, in which K43R and K88R were thought to be the most important mutations blocking SM binding sites, as described in *Mycobacterium tuberculosis* and other species (Arjomandzadegan & Gravand, 2015). Followed by site-directed mutagenesis system, we successfully obtained two derivatives 1401D43 and 1401D88 which are completely resistant to SM from two clinical *H. parasuis* isolates and wild type SC1401. These mutants facilitate our later conjugation process of creating unmarked *fox* and *arcA* knock-out mutants in 1401D88. The hot mutation sites in *H. parasuis* also indicates that the K → R mutations in 43rd and 88th codons in *rpsL*, which encodes the highly conserved RpsL protein of the ribosomal accuracy centre (Toivonen, Boocock & Jacobs, 1999), have a greater propensity to confer drug resistance to streptomycin than *rrs* and other mutation sites. Since we failed to obtain a derivative with simultaneous mutations in both sites, we postulated that mutation at one codon is enough to confer complete SM resistance (Table 1), with no need for generating both point mutations in a same strain, or that mutations at both sites simultaneously are not efficient. It is noteworthy that the unmarked mutation system in this report is not a strictly markerless mutation method from the point of the whole genome, since we have introduced a SM resistance in this strain through the mutation in *rpsL* gene.

We used genomic DNA of 1401D43, 1401D88, and SC1401 $\Delta$ *htrA*, as well as plasmid DNA pKBHK to transform wild type SC1401 to test the transformation frequencies. The results demonstrate that the transformation frequencies of genomic DNA of 1401D43 and 1401D88 occur at a high level (about  $2.458 \times 10^{-3}$ ), nearly four times higher than that of SC1401 $\Delta$ *htrA* (about  $4.748 \times 10^{-4}$ ). We postulated that point mutations that don't generate negative effects on growth will presumably to integrate into host's genome more efficiently under the RecA-driven homologous recombination. Since the genomic DNA of 1401D43 and 1401D88 can be actively and efficiently taken up by natural competent cells, DNA with antibiotic-resistant markers could be used to screen out natural competent bacterium or to verify natural transformation in HGT, since *rpsL* gene is highly conserved in *H. parasuis*, but not *htrA* gene, which is a differentially expressed gene (DEGs) in strains isolated from the lung of a diseased pig and an avirulent strain isolated from the nasal swab of a healthy pig (Zhang et al., 2014b).

As a whole, a modification of an EMS (ethyl methane sulfonate) system was applied to generate streptomycin (SM) resistant in *H. parasuis* based on its efficient mutagenesis which may exhibit stochasticity in the molecular processes at the single-cell level (Parkhomchuk et al., 2009). DNA sequencing was performed to identify a mutation occurring in *rpsL* at codons 43rd and 88th that confer SM-resistance. Site-direct mutagenesis was applied to generate the same point mutation in clinical *H. parasuis* isolates SC1401. The derived *H. parasuis* 1401D88 bearing single point mutation in *rpsL* gene was used to generate a

double mutation ( $\Delta tfox\Delta arcA$ ). Specially, competence-specific *tfox* gene (the orthologue of *sxy* in *H. influenzae*) was reported to be one of the most important regulons controlling natural competence in *H. influenzae* (Cameron et al., 2008), *Aggregatibacter* (formerly known as *Actinobacillus*) *actinomycetemcomitans* (Bhattacharjee, Fine & Figurski, 2007) and *V. cholerae* (Meibom et al., 2005; Johnston et al., 2014). Another gene, *arcA*, encoding ArcA, a response regulator, and a sensor kinase ArcB, is one of the three two-component signal transduction systems (TCSTS) found in *H. parasuis*. We have previously demonstrated that *arcA* gene contributes to the serum resistance and virulence in this species in a previous study (Ding et al., 2016). Furthermore, the genomic DNA of the highly resistant derivatives 1401D43 and 1401D88 could be used as a useful tool to verify natural transformation, thus enabling us to verify naturally competent cells or study horizontal gene transfer (HGT).

## CONCLUSIONS

In summary, our new novel development of EMS-induced SM-resistant method and site-directed point mutations in *H. parasuis* provided a new avenue for genetic manipulation in this bacterium. We have established a discontinuous induction method of EMS for triggering somatic hypermutation in random sites in *H. parasuis*, and successfully screened out hot point mutations in *rpsL* which confer complete SM resistance upon transformants. The DNA containing the desired mutations could be used as a positive selection marker to create an unmarked in-frame *tfox* and *arcA* double knock-out mutants which cannot be established through natural transformation. Finally, we confirmed that the genomic DNA harboring *rpsL* hot mutations was effective and efficient in verifying natural transformation, which will facilitate the identification of natural competent cells and further studies on the mechanism of natural competence in *H. parasuis*.

## ADDITIONAL INFORMATION AND DECLARATIONS

### Funding

This work was supported by the Ministry of Agriculture of the People's Republic of China (MOA) (201303034). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

### Grant Disclosures

The following grant information was disclosed by the authors:  
Ministry of Agriculture of the People's Republic of China (MOA): 201303034.

### Competing Interests

The authors declare there are no competing interests.

### Author Contributions

- Ke Dai conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper.
- Xintian Wen conceived and designed the experiments, wrote the paper.



- Yung-Fu Chang conceived and designed the experiments, analyzed the data, wrote the paper, reviewed drafts of the paper.
- Sanjie Cao, Qin Zhao and Xiaobo Huang performed the experiments.
- Rui Wu, Yong Huang, Qigui Yan and Xinfeng Han analyzed the data.
- Xiaoping Ma prepared figures and/or tables.
- Yiping Wen prepared figures and/or tables, reviewed drafts of the paper.

### Data Availability

The following information was supplied regarding data availability:

The raw data has been provided as a [Supplemental File](#).

### Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.4253#supplemental-information>.

## REFERENCES

- Arjomandzadegan M, Gravand S. 2015.** Analysis of *rpsL* and *rrs* genes mutations related to streptomycin resistance in Mdr and Xdr clinical isolates of *Mycobacterium tuberculosis*. *Tuberk Toraks* **63**:235–242 DOI [10.5578/tt.6474](https://doi.org/10.5578/tt.6474).
- Bhattacharjee MK, Fine DH, Figurski DH. 2007.** *tfox* (*sxy*)-dependent transformation of *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*. *Gene* **399**:53–64 DOI [10.1016/j.gene.2007.04.026](https://doi.org/10.1016/j.gene.2007.04.026).
- Bigas A, Garrido ME, De Rozas AM, Badiola I, Barbe J, Llagostera M. 2005.** Development of a genetic manipulation system for *Haemophilus parasuis*. *Veterinary Microbiology* **105**:223–228 DOI [10.1016/j.vetmic.2004.10.015](https://doi.org/10.1016/j.vetmic.2004.10.015).
- Cameron AD, Volar M, Bannister LA, Redfield RJ. 2008.** RNA secondary structure regulates the translation of *sxy* and competence development in *Haemophilus influenzae*. *Nucleic Acids Research* **36**:10–20 DOI [10.1093/nar/gkm915](https://doi.org/10.1093/nar/gkm915).
- Ding L, Wen X, He L, Yan X, Wen Y, Cao S, Huang X, Wu R, Wen Y. 2016.** The *arca* gene contributes to the serum resistance and virulence of *Haemophilus parasuis* serovar 13 clinical strain EP3. *Veterinary Microbiology* **196**:67–71 DOI [10.1016/j.vetmic.2016.10.011](https://doi.org/10.1016/j.vetmic.2016.10.011).
- Heuermann D, Haas R. 1998.** A stable shuttle vector system for efficient genetic complementation of *Helicobacter pylori* strains by transformation and conjugation. *Molecular and General Genetics* **257**:519–528 DOI [10.1007/s004380050677](https://doi.org/10.1007/s004380050677).
- Huang J, Wang X, Cao Q, Feng F, Xu X, Cai X. 2016.** ClpP participates in stress tolerance and negatively regulates biofilm formation in *Haemophilus parasuis*. *Veterinary Microbiology* **182**:141–149 DOI [10.1016/j.vetmic.2015.11.020](https://doi.org/10.1016/j.vetmic.2015.11.020).
- Johnston C, Martin B, Fichant G, Polard P, Claverys JP. 2014.** Bacterial transformation: distribution, shared mechanisms and divergent control. *Nature Reviews. Microbiology* **12**:181–196 DOI [10.1038/nrmicro3199](https://doi.org/10.1038/nrmicro3199).

- Kielstein P, Rapp-Gabrielson V. 1992.** Designation of 15 serovars of *Haemophilus parasuis* on the basis of immunodiffusion using heat-stable antigen extracts. *Journal of Clinical Microbiology* **30**:862–865.
- Kim IK, Jeong WK, Lim SH, Hwang IK, Kim YH. 2011.** The small ribosomal protein S12P gene *rpsL* as an efficient positive selection marker in allelic exchange mutation systems for *Corynebacterium glutamicum*. *Journal of Microbiological Methods* **84**:128–130 DOI [10.1016/j.mimet.2010.10.007](https://doi.org/10.1016/j.mimet.2010.10.007).
- Li J, Yuan X, Xu L, Kang L, Jiang J, Wang Y. 2016a.** Efficient construction of *Haemophilus parasuis* mutants based on natural transformation. *Canadian Journal of Veterinary Research* **80**:281–286.
- Li Y, Cao S, Zhang L, Lau GW, Wen Y, Wu R, Zhao Q, Huang X, Yan Q, Huang Y, Wen X. 2016b.** A TolC-like protein of *Actinobacillus pleuropneumoniae* is involved in antibiotic resistance and biofilm formation. *Frontiers in Microbiology* **7**:1618–1630 DOI [10.3389/fmicb.2016.01618](https://doi.org/10.3389/fmicb.2016.01618).
- Meibom KL, Blokesch M, Dolganov NA, Wu CY, Schoolnik GK. 2005.** Chitin induces natural competence in *Vibrio cholerae*. *Science* **310**:1824–1827 DOI [10.1126/science.1120096](https://doi.org/10.1126/science.1120096).
- Oliveira S, Pijoan C. 2004.** *Haemophilus parasuis*: new trends on diagnosis, epidemiology and control. *Veterinary Microbiology* **99**:1–12 DOI [10.1016/j.vetmic.2003.12.001](https://doi.org/10.1016/j.vetmic.2003.12.001).
- Oswald W, Tonpitak W, Ohrt G, Gerlach G. 1999.** A single-step transconjugation system for the introduction of unmarked deletions into *Actinobacillus pleuropneumoniae* serotype 7 using a sucrose sensitivity marker. *FEMS Microbiology Letters* **179**:153–160 DOI [10.1111/j.1574-6968.1999.tb08721.x](https://doi.org/10.1111/j.1574-6968.1999.tb08721.x).
- Parkhomchuk D, Amstislavskiy V, Soldatov A, Ogryzko V. 2009.** Use of high throughput sequencing to observe genome dynamics at a single cell level. *Proceedings of the National Academy of Sciences of the United States of America* **106**:20830–20835 DOI [10.1073/pnas.0906681106](https://doi.org/10.1073/pnas.0906681106).
- RStudio Team. 2015.** RStudio: Integrated Development for R. Boston: RStudio, Inc. Available at <http://www.rstudio.com/>.
- Reyrat JM, Pelicic V, Gicquel B, Rappuoli R. 1998.** Counterselectable markers: untapped tools for bacterial genetics and pathogenesis. *Infection and Immunity* **66**:4011–4017.
- Schatz A, Bugie E, Waksman SA. 2005.** Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. 1944. *Clinical Orthopaedics and Related Research* **437**:3–6.
- Seitz P, Blokesch M. 2013.** Cues and regulatory pathways involved in natural competence and transformation in pathogenic and environmental Gram-negative bacteria. *FEMS Microbiology Reviews* **37**:336–363 DOI [10.1111/j.1574-6976.2012.00353.x](https://doi.org/10.1111/j.1574-6976.2012.00353.x).
- Shah SN, Gong ZH, Arisha MH, Khan A, Tian SL. 2015.** Effect of ethyl methyl sulfonate concentration and different treatment conditions on germination and seedling growth of the cucumber cultivar Chinese long (9930). *Genetics and Molecular Research* **14**:2440–2449 DOI [10.4238/2015.March.30.2](https://doi.org/10.4238/2015.March.30.2).

- Shang RF, Wang GH, Xu XM, Liu SJ, Zhang C, Yi YP, Liang JP, Liu Y. 2014. Synthesis and biological evaluation of new pleuromutilin derivatives as antibacterial agents. *Molecules* **19**:19050–19065 DOI [10.3390/molecules191119050](https://doi.org/10.3390/molecules191119050).
- Sinha S, Cameron AD, Redfield RJ. 2009. Sxy induces a CRP-S regulon in *Escherichia coli*. *Journal of Bacteriology* **191**:5180–5195 DOI [10.1128/JB.00476-09](https://doi.org/10.1128/JB.00476-09).
- Sinha S, Mell JC, Redfield RJ. 2012. Seventeen Sxy-dependent cyclic AMP receptor protein site-regulated genes are needed for natural transformation in *Haemophilus influenzae*. *Journal of Bacteriology* **194**:5245–5254 DOI [10.1128/JB.00671-12](https://doi.org/10.1128/JB.00671-12).
- Skorupski K, Taylor RK. 1996. Positive selection vectors for allelic exchange. *Gene* **169**:47–52 DOI [10.1016/0378-1119\(95\)00793-8](https://doi.org/10.1016/0378-1119(95)00793-8).
- Suriyanarayanan B, Lakshmi PP, Santhosh RS, Dhevendaran K, Priya B, Krishna S. 2016. Streptomycin affinity depends on 13 amino acids forming a loop in homology modelled ribosomal S12 protein (*rpsL* gene) of *Lysinibacillus sphaericus* DLSL5 associated with marine sponge (*Tedania anhelans*). *Journal of Biomolecular Structure and Dynamics* **34**:1190–1200 DOI [10.1080/07391102.2015.1073633](https://doi.org/10.1080/07391102.2015.1073633).
- Timms AR, Steingrimsdottir H, Lehmann AR, Bridges BA. 1992. Mutant sequences in the *rpsL* gene of *Escherichia coli* B/r: mechanistic implications for spontaneous and ultraviolet light mutagenesis. *Molecular and General Genetics* **232**:89–96 DOI [10.1007/BF00299141](https://doi.org/10.1007/BF00299141).
- Toivonen JM, Boocock MR, Jacobs HT. 1999. Modelling in *Escherichia coli* of mutations in mitoribosomal protein S12: novel mutant phenotypes of *rpsL*. *Molecular Microbiology* **31**:1735–1746 DOI [10.1046/j.1365-2958.1999.01307.x](https://doi.org/10.1046/j.1365-2958.1999.01307.x).
- Tsai YK, Liou CH, Lin JC, Ma L, Fung CP, Chang FY, Siu LK. 2014. A suitable streptomycin-resistant mutant for constructing unmarked in-frame gene deletions using *rpsL* as a counter-selection marker. *PLOS ONE* **9**:e109258 DOI [10.1371/journal.pone.0109258](https://doi.org/10.1371/journal.pone.0109258).
- Wang F, Shen H, Guan M, Wang Y, Feng Y, Weng X, Wang H, Zhang W. 2011. High-resolution melting facilitates mutation screening of *rpsL* gene associated with streptomycin resistance in *Mycobacterium tuberculosis*. *Microbiological Research* **166**:121–128 DOI [10.1016/j.micres.2010.02.001](https://doi.org/10.1016/j.micres.2010.02.001).
- Xu Z, Yue M, Zhou R, Jin Q, Fan Y, Bei W, Chen H. 2011. Genomic characterization of *Haemophilus parasuis* SH0165, a highly virulent strain of serovar 5 prevalent in China. *PLOS ONE* **6**:e19631 DOI [10.1371/journal.pone.0019631](https://doi.org/10.1371/journal.pone.0019631).
- Zhang B, Yu Y, Zeng Z, Ren Y, Yue H. 2014a. Deletion of the *rfaE* gene in *Haemophilus parasuis* SC096 strain attenuates serum resistance, adhesion and invasion. *Microbial Pathogenesis* **74**:33–37 DOI [10.1016/j.micpath.2014.07.006](https://doi.org/10.1016/j.micpath.2014.07.006).
- Zhang L, Li Y, Dai K, Wen X, Wu R, Huang X, Jin J, Xu K, Yan Q, Huang Y, Ma X, Wen Y, Cao S. 2015. Establishment of a successive markerless mutation system in *Haemophilus parasuis* through natural transformation. *PLOS ONE* **10**:e0127393 DOI [10.1371/journal.pone.0127393](https://doi.org/10.1371/journal.pone.0127393).
- Zhang L, Li Y, Wen Y, Lau GW, Huang X, Wu R, Yan Q, Huang Y, Zhao Q, Ma X, Wen X, Cao S. 2016. HtrA is important for stress resistance and virulence in *Haemophilus parasuis*. *Infection and Immunity* **84**(8):2209–2219.

- Zhang L, Mah TF. 2008.** Involvement of a novel efflux system in biofilm-specific resistance to antibiotics. *Journal of Bacteriology* **190**:4447–4452 DOI [10.1128/JB.01655-07](https://doi.org/10.1128/JB.01655-07).
- Zhang L, Wen Y, Li Y, Wei X, Yan X, Wen X, Wu R, Huang X, Huang Y, Yan Q. 2014b.** Comparative proteomic analysis of the membrane proteins of two *Haemophilus parasuis* strains to identify proteins that may help in habitat adaptation and pathogenesis. *Proteome Science* **12**:38–48 DOI [10.1186/1477-5956-12-38](https://doi.org/10.1186/1477-5956-12-38).
- Zhou Q, Feng S, Zhang J, Jia A, Yang K, Xing K, Liao M, Fan H. 2016.** Two glycosyltransferase genes of *Haemophilus parasuis* SC096 implicated in lipooligosaccharide biosynthesis, serum resistance, adherence, and invasion. *Frontiers in Cellular and Infection Microbiology* **6**:100–109.