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

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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, $CH_3SO_2OC_2H_5$) . 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 to perform homologous recombination through conjugation and successfully generated a double unmarked in-frame targeted mutant 1401D88△*tfox*△*arcA*. Combined with 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.

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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, $CH_3SO_2OC_2H_5$). 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 to perform homologous recombination through conjugation and successfully generated a double unmarked in-frame targeted mutant 1401D88Δ*tfox*Δ*arcA*. Combined with 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.

Key Words:

Haemophilus parasuis, EMS, *rpsL*, streptomycin resistance, natural transformation, point mutation

35

36 **1. Introduction**

37 *Haemophilus parasuis* (*H. parasuis*, HPS) is a Gram-negative, non-spore-forming,
38 pleomorphic, NAD-dependent opportunistic bacterium, and a major causative agent of Glässer's
39 disease. This illness is characterized by polyarthritis, fibrinous polyserositis and meningitis in pigs,
40 producing significant mortality and morbidity in pig farms and leading to serious economic losses
41 in the pork industry throughout the world (Yue et al., 2009;Zhang et al., 2016b). At least fifteen
42 serotypes of varying virulence levels have been identified in this species (Kielstein and Rapp-
43 Gabrielson, 1992). However, numerous gaps in our knowledge of molecular mechanisms of
44 invading internal organs causing local and disseminated infection still remain at the present time
45 (Zhou et al., 2016).

46 The conjugation process of creating markerless mutation is employed in an array of bacteria,
47 which serves as a useful tool of constructing multi-gene scarless knock-outs. This facilitates in the
48 construction of live attenuated vaccine strains and allows researchers to dissect the molecular
49 mechanisms of virulence factors of *H. parasuis* and other bacterium (Heuermann and Haas,
50 1998;Oswald et al., 1999). A prerequisite for this process includes a marker-containing strain
51 (Reyrat et al., 1998); a streptomycin resistance marker based on *rpsL* or *rrs* mutations is one of the
52 most frequently used phenotypes (Tsai et al., 2014).

53 Streptomycin (SM), belonging to aminoglycoside antibiotic, acts on the ribosome, inhibiting
54 the translation of mRNA and therefore disrupting protein synthesis (Schatz et al., 2005). High-
55 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 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 and Taylor, 1996;Kim et al., 2011;Tsai et al., 2014). However, spontaneous or UV-induced mutants of streptomycin resistance in *H. parasuis* probably occurs at a fairly low rate in *H. parasuis;* SM mutant generation in this organism is very inefficient using these strategies (data not shown). Moreover, the molecular mechanism of SM resistance is still unclear in this species.

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;Zhang et al., 2015). 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 (Δ*tfox*Δ*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 et al., 2007) and *V. cholerae* (Meibom et al., 2005;Johnston et al., 2014), et al. 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 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 horizonal gene transfer (HGT). 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. In the light of these findings, we generated a double-gene mutant in *H. parasuis* and used it to perform natural transformation frequency analysis.

2. Material and methods

2.1. Bacteria strains, plasmids and culture conditions

The bacteria strains, plasmids and primers used in this study are listed in **Table S1** and **Table S2**. Plasmids were propagated in *E. coli* DH5α or S17-1 (λpir) and grown in liquid Luria-Bertani (LB, Difco, USA) medium or on LB agar (Invitrogen, China) plate. When required, the medium was supplemented with kanamycin (Kan; 50 μg ml^{-1}) or ampicillin (Amp; 100 μg ml^{-1}) from Sigma-Aldrich, USA. *H. parasuis* was routinely cultured in Tryptic Soy Broth (TSB, Difco, USA) or on Tryptic Soy agar (TSA, Difco, USA) supplemented with 5% inactivated bovine serum (Solarbio, China) and 0.1% (*w/v*) nicotinamide adenine dinucleotide (NAD, Sigma-Aldrich, USA) (TSB++ and TSA++). Where necessary, the media were supplemented with streptomycin (SM; 25 μg ml^{-1}) or kanamycin (Kan; 50 μg ml^{-1}). Unless otherwise stated, all strains were grown at 37℃.

98

99 *2.2. DNA Manipulations*

100 Bacterial genomic DNA was extracted using a TIANamp Bacteria DNA Kit (TIANGEN,
101 China). Small-scale plasmid DNA preparations were generated using a E.Z.N.A™ Plasmid
102 Miniprep Kit (Omega, USA). The DNA fragments were amplified in a C1000™ Thermal Cycler
103 using the 2×Taq Plus Master Mix (Vazyme, China) or I-5™ 2×High Fidelity Master Mix
104 (MCLAB, USA). Purification of DNA fragments from the PCR reaction and the restriction digests
105 were performed using the DNA Fragment Purification Kit Ver.4.0 (TaKaRa, Japan). In-fusion
106 segments were recombined to linearized pK18mobsacB (EcoRI/BamHI) using a ClonExpress Ⅱ
107 One Step Cloning Kit (Vazyme, China). Restriction enzymes were purchased from TaKaRa. Point
108 mutations were performed according to manual of *Fast* mutagenesis system Kit (TransGen
109 Biotech, China).

110

111 *2.3. Antibiotic and Ethyl Methane Sulfonate (EMS) Susceptibility Assays*

112 Assays on the minimal inhibitory concentration were carried out as described by Li. (Li et al.,
113 2016b). Briefly, overnight-grown bacteria were grown in TSB++ with twofold serially diluted SM
114 (from 2 μg/mL to 128 μg/mL; MIC-S) in a 96-well microtiter plate. The plates were incubated at
115 37℃ for 24 h, after which, the results were scored for growth or no growth. The experiments were
116 independently performed at least three times in triplicate. The MIC of EMS (from 0.5mM to 40
117 mM; MIC-E) was analyzed in sterilized tubes. The results were also scored for growth or no
118 growth. Moreover, the MIC-S of EMS induced SC1401 derivatives were also determined using

119 above method.

120 The minimal bactericidal concentrations of EMS for *H. parasuis* (MBC-E) were determined
121 using the method modified from Li and Zhang (Zhang and Mah, 2008;Li et al., 2016b). Overnight-
122 grown *H. parasuis* in sterilized tubes were exposed to different concentration of EMS (from
123 0.5mM to 40 mM) for at least 12 h. Then, fresh TSB++ was used to replace the EMS medium and
124 incubated for additional 16 h. The viability of bacteria was assessed by transferring a spot of the
125 culture onto a TSA++ plate and incubation for 16 h. The experiments were independently
126 performed at least three times in triplicate. Bacteria were serially passaged in TSB++ with MIC-E
127 of EMS for five more generations for further determining the optimum inducing concentration of
128 EMS.

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

136

137 *2.4. Ethyl Methane Sulfonate Discontinuously Inducing Method*

138 Wild type SC1401 (deposited in GenBank under the accession NO. NZ_CP015099.1), which
139 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℃ with agitation for 12h. 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 10mM 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.

2.5. 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, 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.

2.6. 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 SC1401using 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×*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 $CaCl_2$ 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 μF, 200 Ω. The cells were screened by TSA++ containing 25 μg ml^{-1} of SM. The visible single colonies were propagated and the DNA extracted and sequenced for further identification.

2.7. 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. 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, 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, China). After confirmation by PCR and sequencing, the resulting plasmid, pkTLR, was mobilized into S17-1 (λpir) by the

182 $CaCl_2$ method.

183

184 *2.8. Construction of Unmarked In-Frame Targeted Mutant 1401D88ΔtfoxΔarcA of H. parasuis*
185 *1401D88*

186 An unmarked *tfox* mutant was constructed using a two-step selective method. DNA was
187 mobilized from E. *coli* S17-1 (λpir) to *H. parasuis* using a filter mating technique adapted by
188 Oswald with some modification (Oswald et al., 1999). Briefly, S17-1 (λpir) harboring pkTLR and
189 1401D88 strains were grown to exponential phase in SOC and TSB++, respectively. The bacteria
190 cultures were centrifuged at 4,350×g for 5 min; the pellet was resuspended in 1mL of TNM-buffer
191 (1 mM Tris-HCl pH 7.2, 10 mM $MgSO_4$, 100 mM NaCl). Aliquots corresponding to 0.5 ml of
192 donor and 1 ml of recipient (each adjusted to an OD600= 1) were mixed and plated onto a
193 nitrocellulose filter membrane (0.45 μm pore size, 2.5 cm diameter, Millipore, Germany). The NC
194 membrane with bacterial mixture was placed onto TSA++ plates and incubated at 30℃ for 12h.
195 Eventually, the cultures were scraped up and spread on selective TSA++ supplemented with 25μg
196 ml^{-1} of SM and 50μg ml^{-1} of Kan and incubated at 37℃ for 24h. The transformants were then
197 examined for sucrose sensitivity by plating on TSA++ supplemented with 10% sucrose (UP,
198 Amresco, USA) and 25μg ml^{-1} of SM. PCR was carried out to confirm the appropriate deletion in
199 colonies resistant to sucrose and sensitive to kanamycin. Afterwards, the mutants were
200 continuously passed to 20 times on TSA++ and primers *tfox*-P1/P2, *tfox*L-P1/*tfox*R-P2 and Hps-
201 P1/P2 were adopted for further confirming stability of the desired mutant.

202 The effectiveness of our novel protocol for generating a double-gene mutant was confirmed

when we further deleted *arcA* gene from 1401D88Δ*tfox*. Likewise, the process was repeated as above, using a different primer set. The upstream and downstream homologous arms regions of *arcA* were amplified using primers *arcA*L-P1/P2 and *arcA*R-P1/P2, respectively. Primers *acrA*-P1/P2, *arcA*L-P1/*arcA*R-P2 and Hps-P1/P2 were adopted for further confirming the stability of the desired mutant.

2.9. 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 mutation were used to verify natural transformation capacity of *H. parasuis* strain SC1401; genomic DNA of SC1401Δ*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 2.8×10^9 cfu/mL). The bacteria were then spotted on TSA++, cultured overnight at 37℃ and resuspended in TSB++ at 20×10^{10} cfu/mL. A 20 μL aliquot of suspension was supplemented with 1 μg of donor DNA. The mixture was incubated for 10 min at 37℃ and then spread in a small area on TSA++. Cultures were incubated for an additional five hours at 37℃ to induce expression of antibiotic resistance. Afterwards, bacteria were harvested and resuspended and plated on TSA++ with SM in concentrations ranging from 25 μg ml-1 to no antibiotic. Bacteria were incubated for 36 h. The visible single colonies were identified by PCR and western blotting.

After incubation, the colonies on TSA++ and on selective plates were counted.

Transformation frequencies were determined from the number of antibiotic-resistant CFU mL^{-1} divided by the total CFU mL^{-1} scored on nonselective agar. Transformation efficiency was evaluated by calculating number of transformed CFU per μg of donor DNA.

2.10. Statistical Analysis

The statistical analysis was performed using R studio (loaded with R V3.3.2) for Windows. 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 (**).

3. RESULTS

3.1. 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 μg ml^{-1}; however, 24 μg ml^{-1} of SM was bactericidal (**Table 1**). Hence 25 μg 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. 1**, we could see flocculent bacteria when the culture was exposed to 10mM of EMS, but not in the TSB++ with 20mM of EMS. Therefore, twenty millimoles per liter of EMS was determined to be the MIC-E of *H. parasuis* SC1401 (**Table 1** and **Fig. 1**).

245 To determine optimal inducing, subinhibitory concentrations of EMS, we tested the minimal
246 bactericidal concentrations (MBC-E). Overnight-grown seed broth was subcultured in fresh
247 TSB++ with different concentration of EMS (from 0.5mM to 40 mM) and subcultured again with
248 TSB++ with no EMS. The results showed that 30mM of EMS was the definitive MBC-E (**Table**
249 **1**).
250
251 *3.2. The EMS-Induced Derivatives of SC1401 and MIC-S Assay*
252 As shown in **Table 1**, thirty mili-moles per liter of EMS has strong bactericidal activity (this
253 concentration was determined to be the MBC-E), whereas 10mM of EMS was shown to be the
254 MIC-E (**Fig. 1** and **Fig. 2**). Therefore, in consideration of maximizing the inductive effect of EMS,
255 we used a discontinuous induction method in our experiments. The culture in a 10mM of EMS-
256 containing TSB++ was transferred into 5 mL of fresh TSB++ without any EMS, in a "Healing
257 Resurgence" step of our protocol. Each generation was plated onto TSA++ with appropriate SM
258 antibiotic and incubated for 24h. In this way, we obtained SM-resistant generations in F7, F9 and
259 F11. Among them, mutations in *rpsL* at codon 43rd (A128G) and 88th (A263G) were confirmed
260 by sequencing.
261 By comparison, these two kinds of mutations conferred complete resistance to SM (**Table 1**).
262 The two derivatives can survive well at a concentration of at least 4,096 μg ml^{-1} of SM. As a
263 control, *H. parasuis* wild type strains SC1401, HPS32, HPS33 can only survive in less than 16 μg
264 ml^{-1} of SM/TSB++. We also performed the Oxford cup assay to visually demonstrate different
265 levels of SM-resistance. As shown in **Fig. 3**, 1401D43 and 1401D88 can form complete bacteria

lawns on TSA++ with both 100µl of 8,192 µg ml^{-1} and 4,096 µg 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.

3.3. 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. 4**), 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 regions. 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

287 which almost fifteen point-mutations have been identified, data not shown), but obviously not a
288 greater factor of inducing the high resistance to SM (**Table 1** and **Fig. 3**). We didn't find any
289 mutations in *rrs* in 1401D88.
290
291 *3.4. Identification of Successful Construction of pkTLR and pkALR*
292 Further investigation of Kan-resistant colonies showed that the in-fusion segments of
293 up/downstream regions of *tfox* was successfully integrated into linearized pK18mobsacB. PCR
294 runs with primers *tfox*L-P1/ *tfox*L-P2, *tfox*R-P1/ *tfox*R-P2 and *tfox*L-P1/*tfox*R-P2 demonstrated
295 that the products were amplified as expected, which was followed by sequencing for further
296 confirmation (**Fig. S3**). The plasmid pkTLR was then used to create unmarked in-frame *tfox*
297 mutations in *H. parasuis*. Likewise, the plasmid pkALR was generated and identified as above.
298 PCR runs were carried out using primers *arcA*L-P1/ *arcA*L-P2, *arcA*R-P1/ *arcA*R-P2 and *arcA*L-
299 P1/*arcA*R-P2.
300
301 *3.5. Construction of an Unmarked Mutation of tfox and arcA Genes in H. parasuis 1401D88*
302 Based on a two-step natural transformation mutagenesis method, we used a 'dual' process
303 with one round of SM selection and a subsequent sucrose counterselection. The in-fusion segment
304 was successfully transferred to the chromosomal DNA of recipient strain and subsequently
305 integrated into the host *tfox* loci through homologous recombination, yielding the *in situ* deletion.
306 Here, the method of EMS induced high level-resistance to SM plays an important role in weeding
307 out donor strain S17-1 (λpir), thus allowing us to generate unmarked mutants in *H. parasuis* that

308 are multi-drug susceptible.

309 The plasmid pkTLR was integrated into the two flanking regions of *tfox* gene at the genomic
310 site, so that the original *tfox* gene was replaced seamlessly. SM and Kan positive selection was
311 highly successful, and the PCR tests validated both the insertion of the kan cassette and *SacB* gene
312 in most of the Kan-resistant clones (**Fig. S4A**). In the second round of homologous recombination,
313 the backbone of pk18mobSacB was removed, followed by sucrose counterselection and
314 kanamycin susceptibility testing. PCRs were carried out to verify the appropriate deletion in
315 colonies resistant to 10% sucrose and 25 μg ml^{-1} of SM but sensitive to Kan (**Fig. S4B**).

316 The second round is to remove *arcA* gene from 1401D88Δ*tfox*. Likewise, pkALR was used
317 to create in-frame *arcA* knock-out mutant. PCR runs were carried out to confirm the appropriate
318 deletion in transformants resistant to sucrose and 25 μg ml^{-1} of SM but sensitive to 20 μg/mL Kan.
319

320 *3.6. Confirmation of 1401D88Δ*tfox*Δ*arcA* by Western Blotting*

321 Western blotting confirmed that *tfoX* and *arcA* can be detected in the whole-cell extract of
322 wild type *H. parasuis* SC1401 and 1401D88, but not in 1401D88Δ*tfox*Δ*arcA* (**Fig. 5**). This result
323 further confirms the successful deletion of *tfox* and *arcA* genes in strain 1401D88.
324

325 *3.7 Verification of Natural Competence*

326 Point mutations in *rpsL* which don't confer negative effect on growth were assumed to be
327 more effective in verifying natural competence. To confirm this hypothesis, we tested the natural
328 frequencies of different donor DNA, including genomic DNA of *H. parasuis* 1401D43, 1401D88,
329 SC1401Δ*htrA* (in which Kan cassette had been stably inserted.) and the plasmid DNA pKBHK

which was previously used to creat *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, SC1401Δ*htrA* genomic DNA after an extended growth period (13 hours) 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 our elucidation highlighted in "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 2.458×10^{-3}) compared to SC1401Δ*htrA* genomic DNA. However, the highest transformation frequency of genomic DNA of SC1401Δ*htrA* reached to 4.748×10^{-4} (**Fig. 6**). 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^R cassette in the *htrA* loci. In independent repeated experiments, more than thirty transformants were directly sequenced for further identification of desired mutations in *rpsL*.

4. 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

351 studies on gene deletion usually based on natural transformation system of highly transformable
352 strains, such as SC1401 (Zhang et al., 2016a), SC096 (Zhang et al., 2014a), EP3 (Ding et al., 2016),
353 CF7066 (Huang et al., 2016). However, the natural transformation technology relies heavily on
354 screening of natural competent cells, which is inefficient since not all bacteria in *H. parasuis* are
355 competent cells, at least under the existent experiment cues and conditions (Seitz and Blokesch,
356 2013;Johnston et al., 2014).

357 In this study, we found that EMS has a characteristic dual function in *H. parasuis*. High
358 concentration of EMS inhibits growth (20mM) or even exhibits a bactericidal effect (30mM),
359 whereas low concentration introduces random somatic hypermutations in genome. Thus we tested
360 the MIC-E and MBC-E and found 10mM of EMS is an optimized concentration for inducing *rpsL*
361 mutations *H. parasuis*. We couldn't obtain SM-resistant derivatives in the earlier attempts using
362 EMS. A discontinuous induction method in which one alternate step labelled "Healing
363 Resurgence" was applied. With this technique, we successfully obtained an array of SM-resistant
364 derivatives, in which K43R and K88R were thought to be the most important mutations blocking
365 SM binding sites, as described in *Mycobacterium tuberculosis* and other species
366 (Arjomandzadegan and Gravand, 2015). Followed by site-directed mutagenesis system, we
367 successfully obtained two derivatives 1401D43 and 1401D88 which are completely resistant to
368 SM from two clinical *H. parasuis* isolates and wild type SC1401. These mutants facilitate our later
369 conjugation process of creating unmarked *tfox* and *arcA* knock-out mutants in 1401D88. The hot
370 mutation sites in *H. parasuis* also indicates that the K→R mutations in 43rd and 88th codons in
371 *rpsL*, which encodes the highly conserved RpsL protein of the ribosomal accuracy centre

(Toivonen et al., 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 in conjunction with another point mutation in a same strain, or mutations at both sites simultaneously is 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 SC1401Δ*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 2.458×10^{-3}), nearly 4 times higher than that of SC1401Δ*htrA* (about 4.748×10^{-4}). We postulated that point mutations that don't generate negative effects on growth will presumably to integrate into host's genome more effficiently 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 genes (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).

5. 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 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*.

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AUTHOR CONTRIBUTIONS

KD, JJ, XW and YFC designed the experiments. SC, XH, RW, and QZ performed the experiments with assistance from QY, YH and XM. KD, YW, YFC and JJ analyzed the data and wrote the paper. All authors read, commented on and approved the final manuscript.

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515 **Table 1 Susceptibility of *H. parasuis* strains to streptomycin (SM) and ethyl methane**
516 **sulfonate (EMS).** [a]MIC-S/E, minimal inhibitory concentration of SM/EMS; MBC-S/E, minimal
517 bactericidal concentrations of SM/EMS. [b]1401D43 and 1401D88 are completely resistant to SM.
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Fig. 1. 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.

Fig. 2. Growth kinetics of different concentration of EMS-exposed cultures of *H. parasuis* SC1401. Overnight cultures of the wild type SC1401were exposed to different concentrations of EMS and cultured. Ten millimoles per liter of EMS shows a significant inhibitory effect on growth of *H. parasuis*, while 20mM of EMS is bactericidal. Error bars indicate a 95% confidence interval.

A

B

Fig. 3. Streptomycin resistance analysis based on Oxford cup assay. Oxford cups containing 8,192 μg ml^{-1} and 4,096 μg ml^{-1} per well of SM were used to analysis bacterial resistance. It demonstrated that **(B)** the derivatives 1401D43 and 1401D88 were completely resistant to SM, whereas **(A)** wild type *H. parasuis* exhibited distinct bacterial inhibition zones.

Fig. 4. 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.

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553 **B**

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555 **Fig. 5. Western blotting analysis of wild type *H. parasuis* and mutant.** The whole-cell lysates
556 of the 1401D88, mutant and SC1401 strains were detected using *tfoX* and *arcA* mouse antiserum,
557 respectively. The derivatives 1401D88 displayed a similar band to the parent strain SC1401, while
558 1401D88Δ*tfox*Δ*arcA* strain did not. Lane 1: 1401D88 strain, Lane 2: 1401D88Δ*tfox*Δ*arcA* strain,
559 Lane 3: SC1401 strain.

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562 **Fig. 6. Natural transformation frequency of different donor DNA.** Lines are achieved by mean
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Table 1(on next page)

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

[a]MIC-S/E, minimal inhibitory concentration of SM/EMS; MBC-S/E, minimal bactericidal concentrations of SM/EMS. [b]1401D43 and 1401D88 are completely resistant to SM.

Object	Test index[a]	Strains[b]				
		SC1401	1401D43	1401D88	HPS32	HPS33
SM	MIC-S(μg/mL)	16	＞4,096	＞4,096	16	16
	MBC-S(μg/mL)	24	＞4,096	＞4,096	28	32
EMS	MIC-E(mM)	20	--	--	--	--
	MBC-E(mM)	30	--	--	--	--

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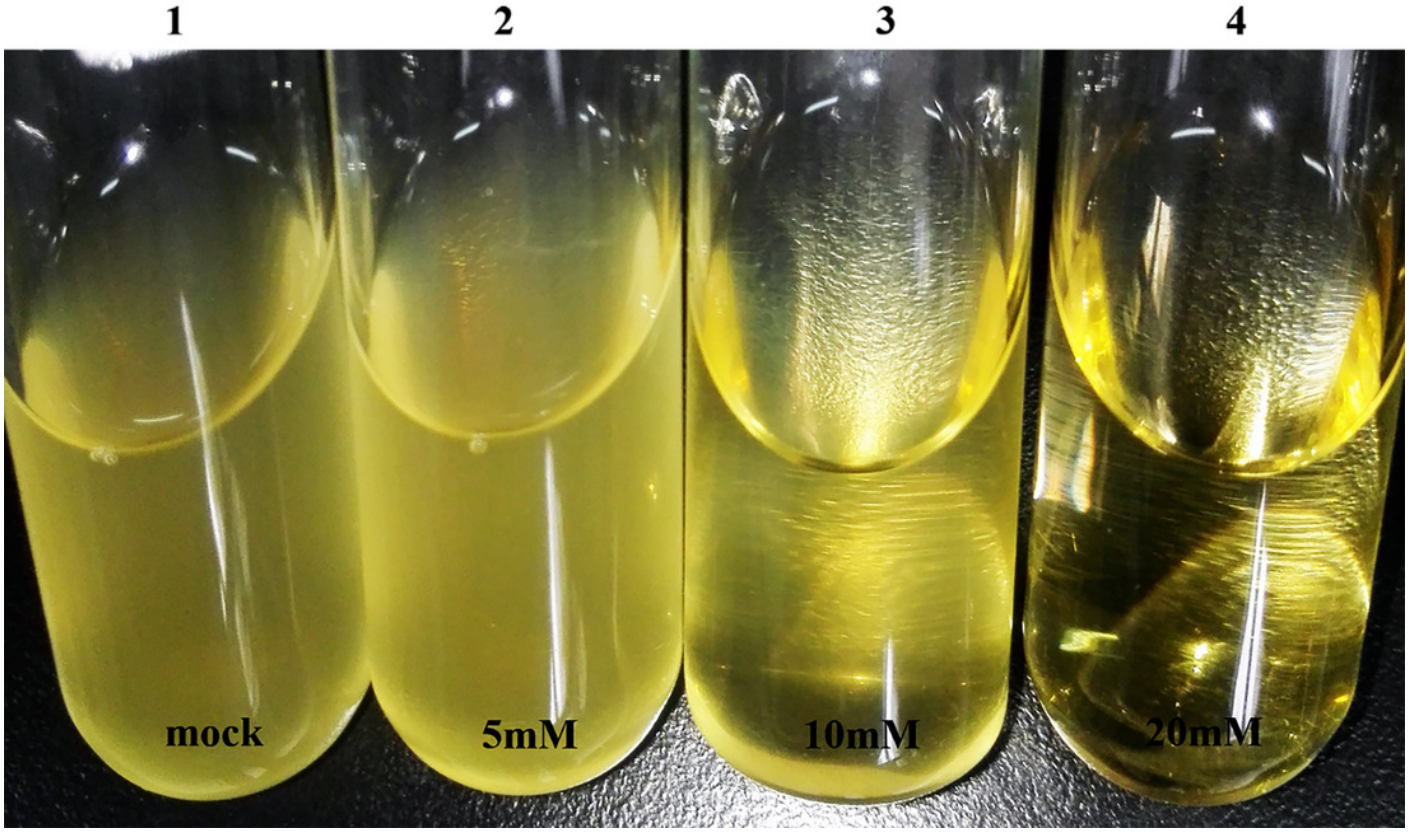

Figure 2(on next page)

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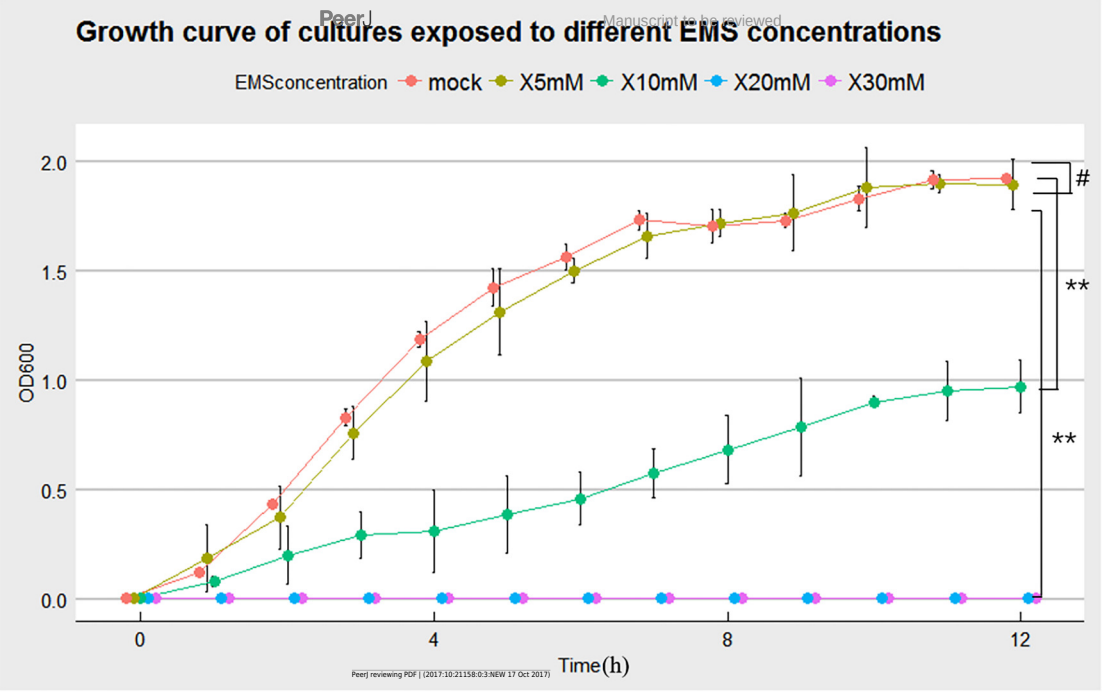

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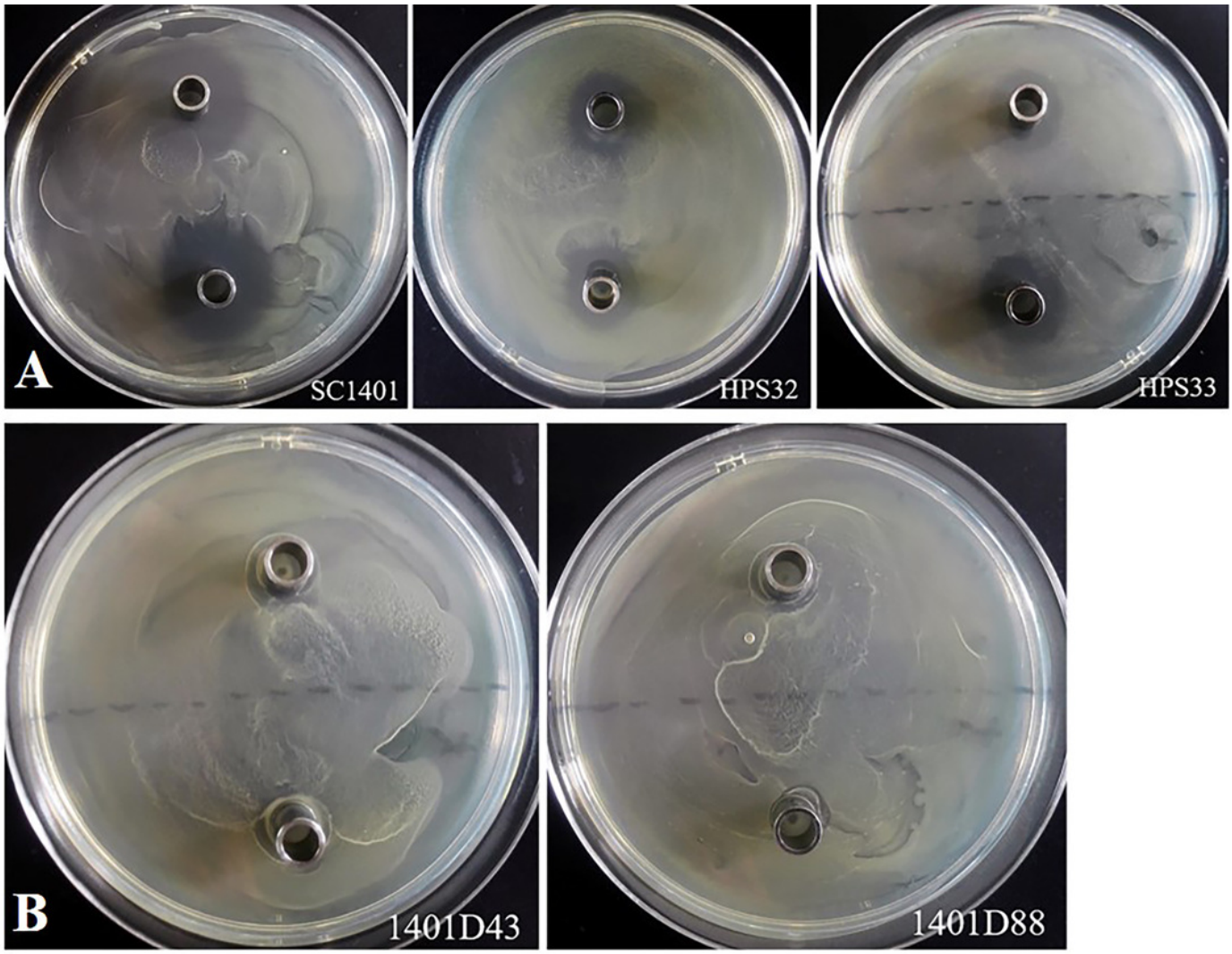

Figure 4(on next page)

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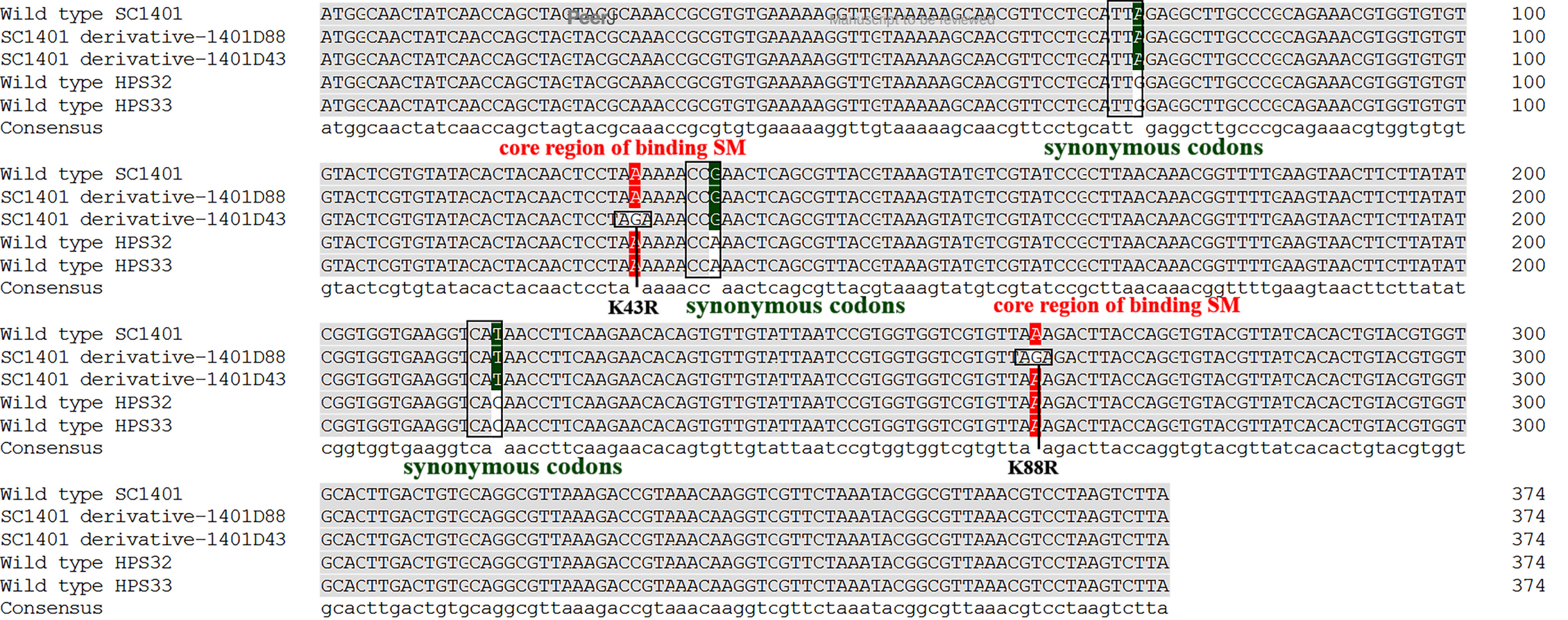

Figure 5

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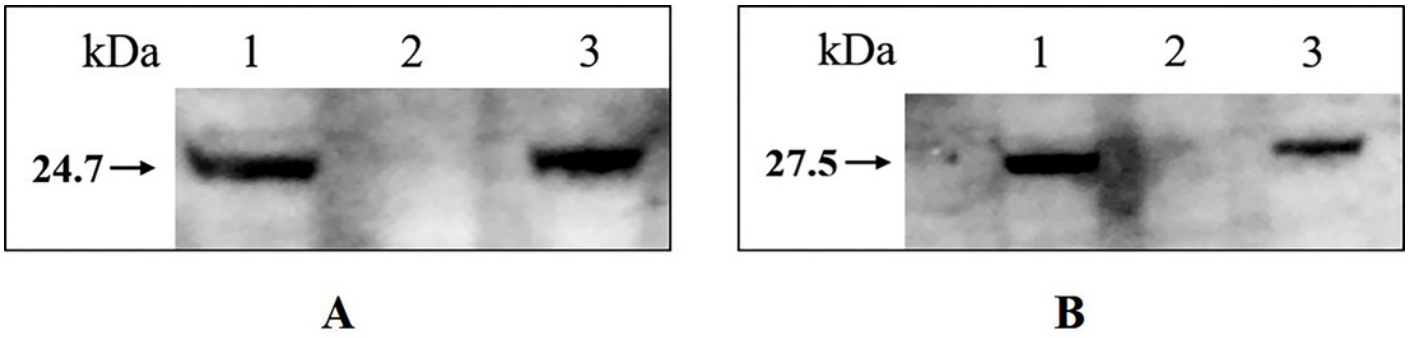

Figure 6(on next page)

Fig. 6. 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.

Transformation frequency analysis of different donor DNA

using different concentrations of DNA to transform wild type SC1401