Serovar-dependent differences in Hfq-regulated phenotypes in *Actinobacillus pleuropneumoniae*

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One-sentence summary: Serovar-dependent differences identified in regulation of complex phenotypes by the RNA chaperone Hfq in the pig pathogen *Actinobacillus pleuroneumoniae* indicate the importance of strain selection and interpretation of results when analysing global gene regulator function.

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Abstract

The RNA chaperone Hfq regulates diverse processes in numerous bacteria. In this study, we compared phenotypes (growth rate, adherence, response to different stress conditions, and virulence in *Galleria mellonella*) of wild-type (WT) and isogenic *hfq* mutants of three serovars (1, 8 and 15) of the porcine pathogen *A. pleuropneumoniae.* Similar growth in rich broth was seen for all strains except Ap1Δ*hfq*, which showed slightly reduced growth throughout the 24 hour time course, and the complemented Ap8Δ*hfq*C mutant had a prolonged lag phase. Differences were seen between the three serovar WT strains regarding adherence, stress response and virulence in *G. mellonella*, and deletion of *hfq* affected some, but not all of these phenotypes, depending on serovar. Complementation by expression of cloned *hfq* from an endogenous promoter only restored some WT phenotypes, indicating that complex regulatory networks may be involved, and that levels of Hfq may be as important as presence/absence of the protein regarding its contribution to gene regulation. Our results support that Hfq is a pleiotropic global regulator in *A. pleuropneumoniae*, but serovar-related differences exist. These results highlight the importance of testing multiple strains/serovars within a given species when determining contributions of global regulators, such as Hfq, to expression of complex phenotypes.

Introduction

The Gram-negative bacterium *Actinobacillus pleuropneumoniae* causes porcine pleuropneumonia, a disease that has a negative economic impact on the worldwide swine industry (Sassu *et al*. 2018). Currently, eighteen serovars are recognized based on

capsular polysaccharides (Bossé *et al*. 2018). All serovars are pathogenic, but some are more virulent than others, e.g. serovar 3 is rarely pathogenic, but serovar 1 is considered of high virulence (Rogers *et al*. 1990; Frey 2011). In part, this is related to the combinations of RTXs toxins (ApxI-III) present in different serovars (Frey 2011). Other virulence factors, some of which also differ depending on serovar, have been reported for *A. pleuropneumoniae* including: capsule, lipopolysaccharide (LPS), fimbriae, outer membrane proteins, iron-binding proteins, and the ability to form biofilms [reviewed in (Bossé *et al*. 2002; Chiers *et al*. 2010)]. In addition, roles in virulence have been indicated for global regulators of gene expression such as RpoE (Bossé *et al*. 2010), HlyX (Buettner *et al*. 2009), ArcA (Buettner *et al*. 2008), and Hfq (Zhou *et al*. 2008; Subashchandrabose *et al*. 2013), the latter being the subject of this study.

Hfq was first identified in 1972 as regulator of phage Qβ RNA replication in *Escherichia coli* (Franze de Fernandez *et al*. 1972). It is now known that, through its interactions with small RNAs (sRNAs), Hfq is a major global regulator of gene expression in a wide variety of bacteria (Vogel and Luisi 2011; Sobrero and Valverde 2012; Feliciano *et al*. 2016; Dos Santos, Arraiano & Andrade 2019). In *E. coli*, deletion of the *hfq* gene results in pleiotropic changes when compared to WT, including increased cell size, reduced growth rate, increased sensitivity to ultraviolet light and other processes (Tsui *et al*. 1994; Kendall *et al*. 2011). A role for Hfq in virulence, as adjudged *in vivo* or by surrogate markers such as tolerance to stress and ability to form biofilms, has been shown for many Gram-negative bacteria including: *Neisseria meningitidis* (Fantappiè *et al*. 2009), *Haemophilus influenzae* (Hempel *et al*. 2013), *Yersinia enterocolitica* (Kakoschke *et al*. 2014), *Brucella melitensis* (Cui *et al*. 2013), *Salmonella enterica* serovar Typhimurium (Behere *et al*. 2016), *Pasteurella multocida*

(Mégroz *et al*. 2016), *Xanthomonas campestris* (Lai *et al*. 2018), and *Bordetella pertussis* (Hayes *et al*. 2020).

With *A. pleuropneumoniae*, it has also been established that Hfq has a role in virulence. Both Zhou *et al*. (2008) and Subashchandrabose *et al.* (2013) demonstrated that *A. pleuropneumoniae hfq* mutants of serovar 1 strains Shope 4074 and AP 93-9, respectively, were less virulent in pigs. In addition, an *hfq* mutant of a clinical serovar 8 isolate, MIDG2331, was attenuated in the *Galleria mellonella* (wax moth) model of infection (Pereira *et al*. 2015). *In vitro*, the AP 93-9 serovar 1 *hfq* mutant was defective in biofilm formation and was more sensitive to superoxide stress (Subashchandrabose *et al*. 2013). In this study, we undertook a comparative analysis of the effect of *hfq* mutagenesis on three different serovars of *A. pleuropneumoniae* to determine if regulation of different Hfq phenotypes is serovar dependent.

Materials and Methods

Bacterial strains, growth conditions and maintenance

The *A. pleuropneumoniae* strains used in this study (listed in Table 1) were routinely grown at 37°C with 5% CO_2 in brain heart infusion (BHI; Difco) broth and agar supplement with 10 µg/mL nicotinamide adenine dinucleotide (NAD; Sigma-Aldrich), and the *E. coli* strains in LB broth and agar. Chloramphenicol (1 or 20 µg/mL, for *A. pleuropneumoniae* and *E. coli*, respectively) or kanamycin (75 µg/mL) was added to the medium when required. Salt-free LB agar (10 g tryptone, 5 g yeast extract and 15 g agar per L) supplemented with 10% filter-sterilized sucrose, 10% horse serum (TCS Biosciences), and 10 µg/mL NAD (Sigma-Aldrich) was used for counter selection of *A.*

pleuropneumoniae mutants, as previously described (Bossé *et al*. 2014). *E. coli* MFD*pir* (Ferrières *et al*. 2010) and Stellar (Clontech) strains were used in conjugation and transformation assays, respectively.

Strain construction

Unless otherwise stated, all PCRs were performed using CloneAmpTM HiFi PCR Premix (Takara), and genomic DNA from the serovar 8 strain MIDG2331 (Bossé *et al*. 2016) was used as the template for amplification of *A. pleuropneumoniae* products for cloning. For direct cloning into the T-vector, pGEM-T (Promega), products amplified with the CloneAmpTM HiFi polymerase were first A-tailed by incubation at 70°C for 30 minutes with 0.2 mM dATP and 5 U of Taq polymerase (Promega), according to manufacturer's instructions. All initial constructs were transformed into *E. coli* Stellar cells (Takara), according to manufacturer's protocol, with selection of clones on media containing chloramphenicol or kanamycin, as appropriate. A description of all primers used in this study is given in Table 2.

The Δ*hfq* and *hfq*::3XFLAG strains of *A. pleuropneumoniae* serovars 8 and 15, and the Ap1Δ*hfqcatsacB* and Ap1*hfq*::3XFLAG*cat* strains of serovar 1, were obtained using the previously described natural transformation technique (Bossé *et al*. 2014). Briefly, the sequence comprising the *hfq* gene, and ~600 bp to either side, was amplified using primers 1 and 2 (Table 2), A-tailed and cloned in pGEM-T (Promega), resulting in pT*hfq*Flank. A selection/counterselection cassette, *catsacB*, was amplified from pUSS*catsac* (Bossé *et al*. 2014) using primers 3 and 4. pT*hfq*Flank was opened by inverse PCR using primers 5 and 6 designed with 15 bp overhangs to allow In-Fusion (Takara) cloning, according to manufacturer's instructions, of the *catsacB* cassette in place of the deleted *hfq* gene to generate plasmid pTΔ*hfqcatsacB*. This plasmid was

122 transformed into *A. pleuropneumoniae* serovars 1, 8, and 15 to obtain Δ*hfqcatsacB*
123 mutants, as previously described (Bossé *et al*. 2014).

124 An unmarked deletion construct was made by amplifying the flanking regions to
125 either side of *hfq*, using primers 1 and 7 for the left flank, and 2 and 8 for the right flank.
126 Primers 7 and 8 contain 15 bp overhangs to allow direct fusion of the two amplicons by
127 over-lap extension (OE) PCR (Bossé *et al*. 2014). The OE PCR product was cloned into
128 pGEM-T (Promega), resulting in pTΔ*hfq*. A construct containing *hfq* with a 3′ fusion to
129 a 3XFLAG tag (3x GAT TAC AAG GAT GAC GAT GAC AGG) was also generated.
130 The 3XFLAG tag was amplified from pDOC-F (accession number GQ889496), a
131 generous gift from S. Wigneshweraraj, using primers 9 and 10. The pT*hfq*Flank
132 construct was opened by inverse PCR using primers 11 and 12, and the 3XFLAG
133 amplicon was inserted by In-Fusion cloning, creating pT*hfq*::3XFLAG.

134 To obtain the unmarked Δ*hfq* and *hfq*::3XFLAG mutant strains, the Δ*hfqcatsacB*
135 mutants were subjected to a second natural transformation with linearized plasmids,
136 either pTΔ*hfq* or pT*hfq*::3XFLAG, with counterselection on LB-SSN plates (Bossé *et*
137 *al*. 2014). As counterselection with the unmarked deletion constructs was not successful
138 with the Ap1Δ*hfqcatsacB* mutant, an alternate construct, pT*hfq*::3XFLAG*cat*, was used
139 to obtain the FLAG-tagged mutant. Primers 13 and 14 were used for amplification of
140 the *cat* cassette of plasmid pUSS*catsac*. The 3xFLAG tag was amplified from pDOC-F
141 using primers 9 and 15. Primers 14 and 15 contain 15 bp overhangs to allow direct
142 fusion of the two amplicons by OE PCR, as above. The pT*hfq*Flank construct was
143 opened by inverse PCR using primers 11 and 12, and the 3xFLAG*cat* amplicon was
144 inserted by In-Fusion cloning, creating pT*hfq*::3XFLAG*cat*. This plasmid was
145 transformed into *A. pleuropneumoniae* serovar 1 to obtain the Ap1Δ*hfq*::3XFLAG*cat*
146 mutant, as previously described (Bossé *et al*. 2014). Deletion of *hfq*, or the presence of

FLAG-tagged *hfq*, in the chromosome of respective mutants was confirmed by PCR and sequencing using primers 1 and 2. RT-PCR analysis using cDNA from both WT and Δ*hfq* mutant strains was performed with primer pairs 16 and 17, 18 and 19, as well as 20 and 21 (for detection of expression of *hflX*, *miaA*, and *hfq*, respectively) in order to confirm that deletion of *hfq* did not affect expression of the flanking genes. As a positive control for each primer pair, gDNA from the WT strain was used. The presence of expressed FLAG-tagged Hfq was confirmed by Western blot using anti-FLAG antibodies (see below).

Hfq promoter analysis and mutation complementation

For a better understanding of the promoter(s) involved in the transcription of the *hfq* gene, a prediction of the *hfq* operon was performed using DOOR (Database for prOkaryotic OpeRons) (Mao *et al*. 2009), followed by prediction of promoters using BPROM (Solovyev *et al*. 2010) and visual analysis of the sequences.

Complementation of the Δ*hfq* mutants was achieved by cloning the *hfq* gene, with three of the predicted endogenous promoters, into the low copy plasmid pMIDG100 (O'Dwyer *et al*. 2004; Bossé *et al*. 2009). Primers 22 and 23 were used to amplify the sequence from 850 bp upstream, to 72 bp downstream, of *hfq*. The vector pMIDG100 was digested with *Eco*RI and *Bst*BI (New England Biolabs) and the 1.2 kb PCR product was inserted using In-Fusion cloning, as above. The plasmid pMIDG_*hfq* was transformed into *E. coli* MFDpir (Ferrières *et al*. 2010) with selection on LB agar containing kanamycin (75 µg/mL), prior to conjugation into the *A. pleuropneumoniae* Δ*hfq* strains to obtain the complemented (Δ*hfq*C) strains. Confirmation of the presence of the gene was performed by PCR and sequencing of the intact gene using the same primers described above.

Growth rate and Hfq expression

For growth curves, the *A. pleuropneumoniae* WT and mutant strains were cultivated in 20 mL of broth in Erlenmeyer flasks incubated at 37°C for 24 hours with agitation (180 rpm). Optical density at 600 nm (OD_{600}) was measured every hour for the first 12 hours, and then at 24 hours, using an Ultrospec 10 (GE Healthcare Life Sciences).

In order to verify expression of Hfq during growth in broth culture, each of the three serovar *hfq*::3XFLAG strains were inoculated into 200 mL of broth (initial OD_{600} 0.01) and then aliquoted into seven flasks of 20 mL each. At time points (1, 2, 3, 4, 6, 8 and 12 hours), one of each serovar culture was centrifuged at 9000x g, and the resulting pellets were re-suspended in 1 mL of lysis buffer (20 mM Tris-HCl, 1 mM EDTA, pH 7.4) and disrupted by mechanical lysis using Matrix B tubes (MP Biomedicals). For each sample, 10 µg of soluble protein were applied to wells of 4-12% NuPAGE Bolt BisTris Plus (Life Technologies – BG04120BOX) gels. Following electrophoretic separation, the proteins were transferred to nitrocellulose membrane (iBlot 2 NC Regular Stacks; Life Technologies - IB23001) using the iBlot 2 system (Life Technologies - IB21001). The membrane was processed, as previously described (Beddek *et al*. 2004), using an anti-FLAG monoclonal (Sigma) as the primary antibody, and detection using ECL Western blotting detection reagents (GE Healthcare) and Hyperfilm ECL (GE Healthcare).

Bacterial adhesion

Bacterial adhesion to three different surfaces was investigated. Adhesion to epithelial A549 cells was determined as previously described by Cuccui *et al*. (2017),

and adherence to polystyrene microtiter plates (Kasvi – K12-096) following growth for 24 h at 37°C was visualized using crystal violet, as described by Kaplan and Mulks (2005). For the third adhesion assay, strains were inoculated in vials containing 1 cm^2 steel coupons, as described previously by Moen *et al*. (2015). Briefly, the vials were incubated at 37°C for a period of 24 h. Cultures were then fixed to the steel coupons with 2.5% glutaraldehyde in 0.05 M phosphate buffered saline (PBS) and dehydrated in a graded ethanol series up to 100%. The cells were dried using a CPD 030 critical point dryer (Bal-Tec) and shadowed with gold using a Sputter Coater (Electron Microscopy Sciences) prior to visualization with a scanning electron microscope (VP1430; LEO).

Stress tolerance

The following agents and their concentrations were used in BHI-NAD agar to investigate the sensitivity of the Δ*hfq* strains to different stress conditions: 1.5% NaCl; pH 6.0 and 6.5 (adjusted using HCl); 1.25 mM H_2O_2; 4% ethanol; and cultivation at 42°C. Bacterial cultures with initial OD_{600} of 1.0 were serially diluted in PBS to 10^{-7}, and 10 µL of each 10-fold dilution were applied on each selective stress agar in square plates (688 102; Greiner Bio-One). As control, cultures were similarly plated on BHI-NAD agar containing no stress agent. All plates were cultured at 37°C, except the temperature stress plate, which was incubated at 42°C. The growth of strains was compared between the control and test plates.

Virulence in *G. mellonella*

The *Galleria mellonella* larvae used in this study were reared in our laboratory, kept at 28°C in darkness and fed an artificial diet. On the day of the experiment, last-

instar larvae, each weighing 250-300 mg, were selected and kept in the same environmental conditions until inoculation, following our previously described methods (Pereira *et al*. 2015; Pereira *et al.* 2018, Blanco *et al*. 2017). Briefly, *A. pleuropneumoniae* cultures were grown to mid-exponential phase and inocula consisting of 10 µl of serially diluted cell suspensions, varying from 10^3 to 10^7 CFU per larva (n=10 larvae per dilution), were injected into the haemocoel of the first right pro-leg. The larvae were incubated at 37 °C, in the dark, and analyzed according to survival at 24, 48, 72 and 96 hours post infection. Larvae were considered as dead if they did not respond to touch stimuli. Survival curves were plotted using the Kaplan-Meier method (Goel *et al*. 2010). For the evaluation of bacterial load, the larval haemolymph was collected at 0, 1, 2, 4 and 24 hours after infection. Thereafter, the CFU/mL were determined. Larvae inoculated with PBS were used as negative controls for the assay.

Statistical Analysis

Data from growth curves and adhesion to A549 cells and polystyrene microtiter plates were analyzed by Tukey's test used to compare means using R v.2.13.0. The differences in *G. mellonella* survival were calculated by using the log-rank test using R v.2.13.0. A $p<0.05$ was considered to be statistically significant. All the assays were done in experimental and biologic triplicates.

Results

Construction *A. pleuropneumoniae hfq* mutants

As previously reported (Subashchandrabose *et al*. 2013), the *hfq* gene in *A. pleuropneumoniae* is located in the *miaA-hfq-hflX* locus, as it is in *E. coli* (Tsui *et al*. 1994). This locus is shown in Fig 1A, with all detected promoters indicated, as well as locations of the priming sites used for PCR amplification of products used to construct the various plasmids. In order to determine the role of Hfq in *A. pleuropneumoniae* serovars 1, 8 and 15, we generated isogenic mutants lacking 220 nucleotides, leaving a truncated *hfq* gene having only 29 nucleotides in the 5′ region and 30 nucleotides in the 3′ region. Clean deletion mutants were generated for serovars 8 and 15, whereas counter selection was not successful with the serovar 1 mutant, leaving the *catsacB* insertion in place of the deleted 220 bases. In order to aid in evaluation of Hfq expression under stress conditions, we also generated isogenic strains where the native *hfq* was replaced with *hfq* additionally encoding a C-terminal 3XFLAG tag (followed by the *cat* gene in the serovar 1 strain). Absence of any polar effects on *miaA* and *hflX* expression in the Δ*hfq* mutants was confirmed by RT-PCR, with representative results for serovar 8 shown in Fig1B. Expression of Hfq by the serovar 1, 8 and 15 Hfq::3XFLAG strains during growth in broth culture was confirmed by Western blotting (Fig1C).

Growth rate and Hfq expression

Only the Ap1Δ*hfq*strain had a reduced growth rate in BHI-NAD, and the complemented Ap8Δ*hfq*C strain had a prolonged lag phase, compared to its isogenic WT strain, which was significant using the Tukey's test ($p<0.05$) (Fig2A). Western blot results for the FLAG-tagged mutants of each serovar showed that Hfq expression was detectable at all time points assayed (Fig 2B), with apparent slight increase in Hfq expression for the Ap8*hfq*::3XFLAG and Ap15*hfq*::3XFLAG, but not Ap1*hfq*::3XFLAG strains over the time course.

Bacterial adhesion

The WT strains of the different serovars tested showed marked differences in adhesion to A549 epithelial cells, with serovar 8 being most, and serovar 15 least, adherent (Fig 3A). Reduction of adherence was significant for the Ap1Δ*hfq* and Ap15Δ*hfq* strains ($p<0.05$), but not Ap8Δ*hfq*, relative to their isogenic WT strains (Fig 3A). Instead of restoring WT levels, expression of *hfq* from the complementation vector further reduced adherence for all serovars (Fig 3A), though the difference was only significant for Ap15Δ*hfq*C ($p<0.05$). All Δ*hfq* mutants had reduced adhesion to polystyrene compared to their WT strains ($p<0.05$) (Fig 3B), with the Ap8Δ*hfq* strain showing the greatest reduction. In contrast to the assay using A549 epithelial cells, all of the complemented strains showed increased adhesion to polystyrene compared to their respective Δ*hfq* mutants, however these increases were not significant and did not restore WT levels. The images of steel coupons obtained by electron microscopy indicated that all Δ*hfq* strains had lower adherence capacity to this surface than their respective WT strains (Fig 3C). This was particularly marked with Ap1Δ*hfq* where there were, in contrast to WT, few adherent cells, with clear complementation in the Ap1Δ*hfq*C strain. Although adherence of the Ap8Δ*hfq* and Ap15Δ*hfq* mutants was not completely abolished, complementation did not restore WT levels (Fig 3C).

Stress tolerance

We investigated the responses of *A. pleuropneumoniae* serovars 1, 8 and 15, and their respective Δ*hfq* mutants, to a variety of stress inducing agents or physical stress (higher temperature) whilst growing on BHI-NAD-agar plates, as shown in Fig 4. The

293 WT strains of serovars 1, 8, and 15 showed different levels of resistance to the different
294 stresses, with serovar 8 being more sensitive to NaCl, and serovar 15 being more
295 resistant to ethanol, but more sensitive to elevated temperature (42°C) and pH 6.0, than
296 the other serovars. Furthermore, the respective Δ*hfq* mutants also showed differences.
297 Unlike Ap8Δ*hfq*, no growth of Ap1Δ*hfq* and Ap15Δ*hfq*was found in the presence of
298 1.25 mM H_2O_2. Compared to the WT strains, all Δ*hfq* mutants were more sensitive to
299 1.5% NaCl, although with differing degrees of growth reduction. Ap8Δ*hfq* and
300 Ap15Δ*hfq* were sensitive to the presence of ethanol, whereas only Ap15Δ*hfq* showed a
301 slight reduction in growth at 42°C. Ap1Δ*hfq* and Ap15Δ*hfq*, in contrast to Ap8Δ*hfq,*
302 were more sensitive to growth at pH 6.5 than their WT strains. Except in the case of the
303 serovar 8 WT and mutant strains grown in the presence of NaCl, restoration of WT
304 levels of growth was achieved by complementation for all other conditions where
305 deletion of *hfq* resulted in increased sensitivity.
306

307 ## Virulence in *G. mellonella*

308 The results of the virulence assay using the *G. mellonella* infection model are
309 shown in Fig 5. The concentration of 1.0 x 10^5 CFU per larva was chosen for graphic
310 representation, as it was found to be the best dose to allow visualization of the
311 differences in the virulence profiles between WT, Δ*hfq* and Δ*hfq*C for each serovar. At
312 24 hours, larvae inoculated with serovar 1 strains showed 4% survival for the WT, 22%
313 for Δ*hfq* and 25% for Δ*hfq*C indicating that the serovar 1 WT was highly virulent in this
314 infection model, and the Δ*hfq* mutant only slightly attenuated compared to the WT
315 (p<0.05), but complementation did not restore the WT level of virulence (Fig 1A).
316 Similar survival rates were found at 96 hours. The serovar 8 WT strain was not as
317 virulent as the serovar 1 WT, with survival of *G. mellonella* of 60% at 24, and 26% at

96 hours (Fig 1A). However, the Ap8Δ*hfq* mutant was fully attenuated, with 100% survival of *G. mellonella* through the 96 hours test period. Partial complementation was seen for Ap8Δ*hfq*C, with 51% survival of *G. mellonella* at 96 hours. The serovar 15 WT did not appear to be virulent in the *G. mellonella* infection model (over 90% survival through 96 hours), and no difference was seen for the AP15Δ*hfq* and AP15Δ*hfq*C strains (Fig 1A). Analysis of bacterial load also showed a similar decrease over time in the larvae infected with the serovar 15 WT, Δ*hfq* and Δ*hfq*C strains (Fig 5B). The Ap1Δ*hfq* and Ap8Δ*hfq* strains both showed a five-log decrease in the number of colonies per larva in the course of 24 hours, and few bacterial (approximately 10^1) cells were observed in the haemolymph after of 24 hours of the experiment (Fig 5B). In contrast, larvae infected with the serovar 1 and 8 WT strains showed an increase of bacterial load between 1 and 4 hours, followed by less than a two-log reduction by 24 hours post-infection. In contrast to the results for the *G. mellonella* survival assay, the Ap1Δ*hfq*C showed partial, whereas the Ap8Δ*hfq*C showed no, complementation in regard to bacterial load.

Discussion

The role of the RNA chaperone Hfq in different bacterial species can be variable. For example, *Francisella novicida* (Chambers and Bender 2011) and *Cronobacter sakazakii* (Kim *et al*. 2015) *hfq* mutants are less resistant to oxidative stress, by contrast *Staphylococcus aureus* mutants are more resistant to oxidative stress (Liu *et al*. 2010). As Hfq mediates the interaction of many sRNAs with their target mRNAs, in some cases leading to repression and in others activation of target gene expression (Vogel and Luisi, 2011; Feliciano *et al*. 2016). The distribution of specific

genes and sRNAs involved in encoding and regulating expression of complex phenotypes such as growth, biofilm formation, stress resistance, and virulence can vary between different serovars/strains of the same species, so it is not surprising to find the effects of global regulators can be significantly strain as well as species dependent.

In this study, we compared the effects of *hfq* mutation in serovars 1 (Shope 4074; reference strain), 8 (MIDG2331; clinical isolate) and 15 (HS143; reference strain) of *A. pleuropneumoniae*, an important swine pathogen for which there are 18 known serovars (Bossé *et al*. 2018) that can vary in their degree of virulence in pigs (Rogers *et al*. 1990; Sassu *et al*. 2018). Serovar 1 isolates, expressing Apx toxins I and II, are typically characterized by high virulence, whereas serovars 8 and 15, expressing ApxII and III, are characterized by moderate virulence (Frey 2011). In addition, factors other than RTX toxins, some of which are serovar specific, also contribute to virulence (Bossé *et al*. 2002; Chiers *et al*. 2010). Initially, our goal had been to characterize the influence of Hfq on several aspects of the physiology of *A. pleuropneumoniae* serovar 8, using MIDG2331 as a model, with serovar 1 and 15 strains used as controls for *hfq* mutation (Subashchandrabose *et al*., 2013) and natural transformation (Bossé *et al.*, 2009), respectively. However, as different phenotypes for the mutants became apparent, along with differences in the virulence profiles and other features of the WT strains, we shifted our efforts towards comparing the differential influence of the lack of Hfq in strains from these distinct serovars.

A previous study by Subashchandrabose *et al*. (2013), characterizing an *hfq* mutant of a clinical serovar 1 strain (AP 93-9), showed a slight reduction in growth rate compared to the WT during cultivation in rich broth, which could be complemented by expression of *hfq* from a plasmid. In our current study, we found similar results for a Δ*hfq* mutant of the serovar 1 reference strain (Shope 4074), however deletion of *hfq* in

367 the serovar 8 and 15 strains tested had no effect on growth in rich broth, indicating a
368 possible serovar-related effect.

369 The majority of clinical isolates of *A. pleuropneumoniae* readily form biofilms,
370 but this phenotype tends to be lost after passage in broth culture, suggesting repression
371 *in vitro* (Kaplan and Mulks 2005). Of the twelve serovar reference strains tested, only
372 the serovar 5b and 11 strains (L20 and 56513, respectively) retained the ability to adhere
373 to glass tubes or polystyrene plates, indicating possible serovar-related differences in
374 regulation of this phenotype (Kaplan and Mulks 2005). Production of a poly-1,6-*N*-
375 acetylglucosamine (PNAG) exopolysaccharide matrix has been shown to be the main
376 contributor to *A. pleuropneumoniae* biofilm formation on abiotic surfaces (Kaplan *et al*.
377 2004; Izano *et al*. 2007), with the O-antigen component of LPS also shown to contribute
378 (Hathroubi *et al*. 2015). Components of LPS, PNAG, pili, outer membrane proteins, and
379 glycoproteins have also been implicated in binding of *A. pleuropneumoniae* to various
380 cell lines (Cuccui *et al*. 2017; Rioux *et al*. 1999; Paradis *et al*. 1994; Van Overbeke *et*
381 *al*. 2002; Auger *et al*. 2009; Li *et al*. 2012; Liu *et al*. 2015; Liu *et al*. 2018), indicating a
382 more complex phenotype than binding to abiotic surfaces.

383 Deletion of *hfq* in the AP 93-9 clinical serovar 1 strain, a strong biofilm former,
384 was shown to reduce expression of *pgaC,* encoding the glycosyltransferase involved in
385 PNAG biosynthesis, and completely abrogated the ability to adhere to polystyrene
386 (Subashchandrabose *et al*. 2013). In our study, we further investigated the contribution
387 of Hfq to regulation of adherence of *A. pleuropneumoniae* to biotic and abiotic surfaces.
388 We found that the serovar 1, 8, and 15 WT strains tested showed different levels of
389 adhesion to various surfaces. The WT serovar 8 clinical isolate (MIDG2331) showed
390 the highest level of adherence to the A549 human alveolar basal epithelial cell line,
391 which we have previously used for *A. pleuropneumoniae* adhesion assays (Cuccui *et al*.

2017), as well as to polystyrene plates. Furthermore, the biofilm formed by the serovar 8 WT on steel coupons showed a more mature 3-D architecture, compared to those of the serovar 1 and 15 WT strains. Although the serovar 15 reference strain showed similar adherence to polystyrene and steel coupons when compared to the serovar 1 reference strain, it also showed the lowest level of adherence to A549 cells.

Deletion of *hfq* resulted in varying degrees of adherence reduction to the different surfaces depending on the serovar. All three serovar Δ*hfq* mutants showed slight, but significant ($p<0.05$), reduction of adherence to polystyrene, with some restoration (not significant) of binding in each following expression of the *hfq* gene from the complementation vector. It is not clear why we did not see complete abrogation of binding to polystyrene with our *hfq* mutants, or full complementation, as was seen in the study by Subashchandrabose *et al* .(2013), but this may have been due to differences in the isolates and/or how the assays were performed. Results of the adhesion assay using steel coupons showed all Δ*hfq* strains had lower adherence capacity to this surface than their respective WT strains, but restoration of the WT adherence phenotype was only seen with the Ap1Δ*hfq*C strain. In the assay using A549 cells, all three Δ*hfq* mutants showed reduced adherence compared to their respective WT strains, but the level of reduction was only significant ($p<0.05$) for the serovar 1 and 15 mutants. As opposed to no, or partial, complementation of the binding phenotypes, each of the three serovar Δ*hfq* mutants expressing the plasmid encoded *hfq* gene showed even further reductions in binding to A549 cells, though this was only significant ($p<0.05$) for the Ap15Δ*hfq*C strain.

It is difficult to determine from the current studies whether the different *in vitro* adhesion phenotypes are due to serovar related differences in encoded adhesion genes, or to changes in gene regulation following passage of clinical isolates in the laboratory,

417 or both. Clearly the clinical serovar 8 WT strain showed the greatest adherence to all of
418 the surfaces tested compared to the WT serovar 1 and 15 reference strains, and deletion
419 of *hfq* reduced adherence in all cases, but to different extents. These data support a role
420 for Hfq in regulating at least some of the gene products contributing to adherence to the
421 different surfaces in each of the serovar strains tested, but other regulators such as RpoE
422 and H-NS (Bossé *et al*. 2010) and the two component systems CpxA/CpxR (Li *et al*.
423 2018) and QseA/QseB (Liu *et al*. 2015) have also been shown to be involved, and their
424 relative contributions to regulating this complex phenotype are unresolved.

425 The ability to respond to and repair damage caused by a variety of stresses is
426 important for the survival of *A. pleuropneumoniae* within its host, especially during
427 acute disease (Sheehan *et al*. 2003; Klitgaard *et al*. 2012). Numerous genes involved in
428 stress response have been identified, and their expression has been shown to be
429 regulated by factors including RpoE and (p)ppGpp (Bossé *et al*. 2010; Li *et al*. 2015). In
430 the study by Subashchandrabose *et al.* (2013), Hfq was shown to contribute to
431 resistance of their clinical serovar 1 isolate to oxidative stress, but other sources of
432 stress were not investigated. Here we have shown that there was variation in the
433 response of the three different serovar WT strains, as well as their Δ*hfq* mutants, to
434 different stressors. For example, the serovar 8 WT was more sensitive to NaCl stress,
435 whereas the serovar 15 WT was more sensitive to heat stress at 42°C, but more resistant
436 to ethanol stress, than the other two WT strains. The Δ*hfq* mutants of serovars 1 and 15,
437 but not serovar 8, were more sensitive to H_2O_2 and pH 6.5 compared to their WT
438 parental strains. In contrast to the adhesion experiments, the stress susceptible
439 phenotypes were complemented by expression of the plasmid-encoded *hfq* gene.

440 Although Subashchandrabose *et al*. (2013) previously reported that deletion of
441 *hfq* in *A. pleuropneumoniae* did not result in increased sensitivity to H_2O_2 or cumene

hydroperoxide, it did increase sensitivity to methyl viologen and potassium tellurite – both known to generate superoxide radicals within bacterial cells. In their study, sensitivity to these agents was tested using a disk diffusion assay, and they used a strong biofilm forming clinical isolate of serovar 1 (AP 93-9) of *A. pleuropneumoniae*. Both of these factors could explain the differences in results found in our current study. Overall, the data indicate that, as for adhesion and biofilm formation, Hfq plays a role in stress resistance, but there are serovar- or even strain-dependent differences in regulation of this complex phenotype.

Finally, we compared virulence of serovar 1, 8 and 15 *A. pleuropneumoniae* WT and Δ*hfq* mutants in the *G. mellonella* infection model that we previously described (Pereira *et al*. 2015). As for the other phenotypes tested, there were variations between the different WT serovars and Δ*hfq* mutant strains with regards to virulence in this model. Since the serovar 15 WT was avirulent under the conditions tested, no difference was seen following deletion of the *hfq* gene in this strain. The serovar 1 WT was the most virulent, but only showed a slight decrease, whereas the moderately virulent serovar 8 WT was completely attenuated following deletion of its *hfq* gene. Furthermore, complementation was not successful for the serovar 1 mutant expressing the plasmid-encoded *hfq* gene, but restored almost full WT level of virulence for the serovar 8 mutant, indicating possible differences in genes (and possible differences in gene regulation) contributing to virulence of these serovars in this infection model.

Complementation of mutated phenotypes using cloned genes expressed from shuttle plasmids is always challenging, as factors including level of expression (due to copy number of plasmid and strength of promoter used for expression) and indirect effects of interaction of the expressed gene with regulatory network(s) can influence the overall success of restoring the WT phenotype. This is especially true for

467 complementation of genes encoding global regulators, such as Hfq. We tried account
468 for possible confounding issues by cloning the *hfq* gene into a low copy number
469 plasmid (pMIDG100), with expression possible from the endogenous sigma 70 and/or
470 sigma E promoters included in the upstream sequence. However, we still found that
471 pMIDG_*hfq* was able to complement some, but not all of the Δ*hfq* mutant phenotypes,
472 and this was sometimes serovar-dependent. In the case of binding to A549 epithelial
473 cells, expression of the plasmid copy of *hfq* resulted in further reductions rather than
474 restoration of WT levels in adherence for all 3 serovar strains. Each of the phenotypes
475 analyzed in this study are complex, and result from coordinated expression of different
476 genes, some of which may be regulated by Hfq-dependent sRNAs and others not.
477 Adding to this complex network, other regulators such as sigma factors and DNA
478 binding proteins may also be involved, and the balance of these factors likely
479 determines the resulting phenotype. Similar observations of partial complementation
480 and/or exacerbation of phenotype have been made by others when expressing *hfq* on
481 plasmids, either from its own promoter or an inducible one (Fantappiè *et al*. 2009;
482 Schilling *et al*. 2009; Bai *et al*. 2010; Chambers *et al*. 2011). It is possible that, even
483 though we deliberately cloned the *hfq* gene along with three possible endogenous
484 promoters, on a low copy number plasmid, the intracellular levels of Hfq were either
485 lower or higher than those present in the WT strains, resulting in differential regulation
486 of genes affecting the different phenotypes.

487 In summary, we found that Hfq contributes to regulation of adhesion to biotic
488 and abiotic surfaces, resistance to various stress conditions, and virulence in a surrogate
489 model of infection, to differing extents in the three serovars of *A. pleuropneumoniae*
490 studied. The full set of genes and sRNAs contributing to each of these phenotypes, and
491 how these differ between serovar/strains of *A. pleuropneumoniae*, remain to be

492 determined. We conclude the need for caution in extrapolating the effects of deletion of
493 global regulators, and *hfq* in particular, to other strains of the same species, especially
494 regarding complex phenotypes.
495

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504 **Table 1. Strains and plasmids used in this study.**

Strains and plasmids	Description	Source or reference
Actinobacillus pleuropneumoniae		
Serotype 1		
Shope 4074 WT	Shope 4074 Wild-type	ATCC 27088
Ap1Δ*hfqcatsacB*	*Δhfq* mutant of Shope 4074	This study
Ap1Δ*hfqcatsacB*C	Complemented strain	This study
Ap1*hfq*::3XFLAG*cat*	WT containing a 3XFLAG tag replacing the last codon of the *hfq* gene.	This study
Serotype 8		
MIDG2331 WT	Serotype 8 clinical isolate from UK	(Bossé *et al*. 2016)
Ap8Δ*hfq*[a]	*Δhfq* mutant of MIDG2331	This study
Ap8Δ*hfq*C	Complemented strain	This study
Ap8*hfq*::3XFLAG	WT containing a 3XFLAG tag replacing the last codon of the *hfq* gene.	This study
Serotype 15		
HS143 WT	HS143 Wild-type	(Blackall *et al*. 2002)
Ap15Δ*hfqcat*	*Δhfq* mutant	This study
Ap15Δ*hq*C	Complemented strain	This study
Ap15*hfq*::3XFLAG	WT containing a 3XFLAG tag replacing the last codon of the *hfq* gene.	This study
Escherichia coli		
Stellar	Competent cell: F–, *endA1*, *supE44*, *thi-1*, *recA1*, *relA1*, *gyrA96*, *phoA*, *Φ80d lacZΔ M15*, Δ (*lacZYA - argF*) *U169*, Δ (*mrr-hsdRMS-mcrBC*), Δ*mcrA*, λ–	Takara
MFDpir	Conjugative cell: MG1655 RP4-2-Tc::[ΔMu1::*aac*(3)IV- Δ*aphA*- Δ*nic35*-ΔMu2::*zeo*] Δ*dapA*::(*erm-pir*) Δ*recA.* Strain used to introduce pMIDG_*hfq* in *A. pleuropneumoniae* Δ*hq* strains.	(Ferrières *et al*. 2010)
Plasmids		
pUSS*catsac*	Template DNA for amplification of *catsacB* cassette which contains DNA uptake sequences for natural transformation into *A. pleuropneumoniae*.	(Bossé *et al*. 2014)
pT*hfq*Flank	Plasmid pGEM-T containing 600 nucleotides upstream *hfq* gene, *hfq* gene and 600 nucleotides downstream *hfq* gene.	This study
pTΔ*hfqcatsacB*	Plasmid pTΔ*hfq* containing the *hfq* gene disrupted by *catsacB* cassette.	This study
pT*hfq*3XFLAG	Plasmid pGEM-T containing the *hfq* gene with a 3XFLAG tag in the region 3´ of the hfq gene.	This study
pT*hfq*3XFLAG*cat*	Plasmid pGEM-T containing the *hfq* gene with a 3XFLAG tag in the region 3´ of the *hfq* gene followed by the *cat* gene.	This study
pT*hfq*::3XFLAG*catsac*B	Plasmid pGEM-T containing the *hfq* gene with a 3XFLAG tag in the region 3´ of the *hfq* gene disrupted by *catsac*B cassette.	This study
pMIDG_*hfq*	pMIDG plasmid (Bossé *et al*., 2009) containing the *hfq* gene under promoter inside of the *mia*A gene. Strain used to complement the Δ*hfq* strains.	This study

505 [a] Although this mutant strain has been used in a previous study by our group (Pereira *et al*., 2015), this is
506 the first description of the generation of the mutation.
507

508 **Table 2. Primers used in this study.**

N°	Primer	Oligonucleotide sequence (5´ to 3´)	Description
1	*hfq*flank_for	TTCCGGTGGAAGTAATTAGCGTAGA	For amplification of the *hfq* cassette.
2	*hfq*flank_rev	ATATCCGCTTTCTGACGAGTTTTGC	
3	*cat*_delta*hfq*	ATCTTTACAAGATCCTACAAGCGGT CGGCAATAAGTTACC	For amplification of *catsacB* cassette containing 15 bp overhangs (underlined) complimentary to pT*hfq*Flank opened by inverse PCR with primers 5 and 6.
4	*sac*_delta*hfq*	CGCAACCGCTTCAACGAATTGCGTG AAGCTCGAGGTATG	
5	delta*hfq*_inv*cat*	TGCCGACCGCTTGTAGGATCTTGTA AAGATTGACCTTTTGC	For inverse PCR amplification of pT*hfq*Flank to remove all but 59 bp of the *hfq* gene and adding 15 bp overhangs (underlined) complementary to the *catsacB* cassette generated with primers 3 and 4.
6	delta*hfq*_inv*sac*	AGCTTCACGCAATTCGTTGAAGCGG TTGCGGATAAAGC	
7	delta*hfq*_1	CGCAACCGCTTCAACGGATCTTGTA AAGATTGACCTTTTGC	For generation of Δ*hfq* construct. Addition of 15 bp overhangs (underlined) allow direct fusion of left flank amplified using primers 1 and 7 to right flank generated using primers 2 and 8.
8	delta*hfq*_2	ATCTTTACAAGATCCGTTGAAGCGG TTGCGGATAAAGC	
9	FLAG_*hfq*	GTTGCGGATAAAGCGGGTACCGAC TACAAAGACCATGAC	For amplification of the 3XxFLAG cassette containing 15 bp overhangs (underlined) complementary to pT*hfq*Flank opened by inverse PCR with primers 11 and 12.
10	FLAG_*hflX*	TTGGTATCTGATCGGCTCCAGCCTA CATTACTATTTATCG	
11	*hfq*_inv1	CGCTTTATCCGCAACCGCTTCAAC	For generation of pT*hfq*::3XFLAG
12	*hfq*_inv2	CCGATCAGATACCAAATACAGATG	
13	*cat*_FLAG	TAATGTAGGCTGGAGGTACAAGCG GTCGGCAATAGTTACC	For amplification of the *cat* cassette containing 15 bp overhangs (underlined) complimentary to 3XFLAG and pT*hfq*Flank opened by inverse PCR with primers 11 and 12.
14	*cat_hflX*	TTGGTATCTGATCGGGAAGTGCGGT ATGCCGTCTGAAC	
15	FLAG_*cat*	GCCGACCGCTTGTACCTCCAGCCTA CATTACTATTTATCG	For amplification, in combination with primer 9, of the 3xFLAG cassette containing 15 bp overhangs (underlined) complementary to *cat* cassette and pT*hfq*Flank opened by inverse PCR with primers 11 and 12.
16	*hflX*_for	CACGAGCTTAGTCCGTCACA	For RT-PCR analysis of *hflX* expression.
17	*hflX*_rev	AATGCTACCCGCTGTATGCT	
18	*miaA*_ for	TAATGGGTCCAACGGCTTCG	For RT-PCR analysis of *miaA* expression.
19	*miaA*_rev	CACTGTTCCAACCTCGCAGCCAAG	
20	EcoRI_*hfq*	GCGCGAATTCAGGAAAAGAAAATG GCAAAAGGTCAATCT	For RT-PCR analysis of *hfq* expression.
21	*hfq*_SacI	GCGCGAGCTCATTATTCCGCTTTAT CCGCAACCGC	
22	*hfq*MIDG_for	GCTCAAGCTTCGAATTCGAGCTTGC CCCTCACCGCTTGATTG	For amplification of *hfq* gene with its own promoter region and containing 15

23	*hfq*MIDG_rev	TTGGGATCTTTCGAAGCGTTTTCAT CTGTATTTGGTATCTG	bp overhangs complementary to pMIDG100 cut with *Eco*RI and *Bst*BI.

509

510 **Figure legends:**

511

512 **Fig 1. Generation and confirmation of *hfq* mutant strains.** (A) Genomic
513 organization of the *miaA*, *hfq* and *hflX* genes in *A. pleuropneumoniae*. Predicted
514 promoter sequences are indicated by the bent arrows labelled P1 to P4, and a predicted
515 transcriptional terminator is indicated by a stem-loop structure downstream of *hfq*. The
516 primers used in mutant construction, cloning and RT-PCR, are represented by arrows
517 bellow their targets, numbered according to their identification in Table 2. (B) RT-PCR
518 analysis of possible polar effects due to deletion of *hfq*, showing representative results
519 for MIDG2331. PCR was performed with the products of cDNA synthesis from RNA
520 template of either the WT or Δ*hfq* strain (as indicated), both with (RT+) and without
521 (RT-) the addition of reverse transcriptase. Note that the genomic DNA (gDNA) control
522 used to confirm primer function and product size for each primer pair was from the WT
523 strain only, thus a product for *hfq* amplification is seen as a comparison for the lack of
524 amplification by RT-PCR from the Δ*hfq* strain. Amplification of the target sequences in
525 *hfq*, *miaA* and *hflX* was achieved with the primer pairs 20/21, 18/19 and 16/17,
526 respectively. M = molecular weight marker (DNA Marker Quick-load 100bp DNA
527 ladder, Neb Biolabs). (C) Western blot showing the detection of the 14 kDa
528 Hfq::3XFLAG protein. For each *hfq*::3XFLAG strain of *A. pleuropneumoniae* serovars
529 1 (Ap1), 8 (Ap8) and 15 (Ap15), ten micrograms of soluble protein from early
530 stationary phase culture were separated by SDS-PAGE and transferred to nitrocellulose
531 membrane for detection using an anti-Flag antibody. The molecular weight marker lane

(M = SeeBlue Plus2; Invitrogen) from the corresponding stained gel is shown next to the blot.

Fig 2. Growth of *A. pleuropneumoniae* WT, *hfq* mutants and complemented strains. (A) Growth curve of *A. pleuropneumoniae* serovars 1, 8 and 15 strains. (B) Hfq::3XFLAG expression analysis during the growth curve of the *A. pleuropneumoniae* strains. WT (wild-type), *hfq*::3XFLAG (strain that express Hfq::3XFLAG), Δ*hfq* (*hfq* mutant), Δ*hfq*C (complemented strain). Error bars are shown for all points in the graphs, but may not be visible in some cases.

Fig 3. Effect of Hfq on adherence of *A. pleuropneumoniae* serovars 1, 8 and 15 to biotic and abiotic surfaces. (A) The adherence to eukaryotic cells. (B) The adherence to polystyrene microplate was examined by crystal violet reading in OD_{600} and the adherence capacity was determined according to WT strains. (C) The adherence to steel coupons was examined by scanning electron microscopy (SEM). Bars: 10 μm. Different letters inside of the Fig represent statistical significance difference among the strains in relation the cell length. The statistical analysis was performed using Tukey's test with $p<0.05$. All the assays were conducted in experimental and biological triplicates. WT (wild-type), Δ*hfq* (*hfq* mutant), Δ*hfq*C (complemented strain).

Fig 4. Effect of Hfq on stress tolerance in *A. pleuropneumoniae* serovars 1, 8 and 15. Exponentially growing *A. pleuropneumoniae* strains (OD_{600} = 1.0; ~ 10^8 cell/mL) were exposed to different stress conditions: oxidative (1.25 mM H_2O_2), osmotic (1.5% NaCl), alcoholic (4% ethanol), temperature (42°C), pH (6.5 and 6.0). As control, the strains were grown in BHI-NAD-agar at 37°C, 5% CO_2 and no stressor agent. The

numbers 1, 2 and 3 indicate the WT (wild-type), Δ*hfq* (*hfq* mutant) and Δ*hfq*C (complemented strain), respectively.

Fig 5. Effect of Hfq on the virulence of *A. pleuropneumoniae* serovars 1, 8 and 15 in *G. mellonella*. (A) Killing was monitored after larval infection with $1x10^5$ CFU of WT, Δ*hfq* and Δ*hfq*C *A. pleuropneumoniae* strains from serovars 1, 8 and 15. The virulence attenuation was verified in the Δ*hfq* strains of serovars 1 and 8 ($p<0,05$). (B) Determining of the bacterial load in *G. mellonella* hemolymph at 0, 1, 2, 4 and 24 hours of the assay of (A) in three biological replicates. Larvae inoculated with PBS 1X were used as negative control. WT (wild-type), Δ*hfq* (*hfq* mutant) and Δ*hfq*C (complemented strain).

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