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1 **An *Actinobacillus pleuropneumoniae* arcA deletion mutant is attenuated and**  
2 **deficient in biofilm formation**

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24

25 **Abstract**

26

27 *Actinobacillus pleuropneumoniae* is a facultative anaerobic pathogen of the  
28 porcine respiratory tract requiring anaerobic metabolic activity for persistence on lung  
29 epithelium. The ArcAB two-component system facilitating metabolic adaptation to  
30 anaerobicity was investigated with regard to its impact on virulence and colonization  
31 of the porcine respiratory tract. Using pig infection experiments we demonstrate that  
32 deletion of *arcA* renders *A. pleuropneumoniae* significantly attenuated in acute  
33 infection and reduced long term survival on unaltered lung epithelium as well as in  
34 sequesters. Contrary to its role in enterobacteria, the deletion of *arcA* in *A.*  
35 *pleuropneumoniae* does not effect growth and survival under anaerobic conditions.  
36 Instead, other than the parent strain *A. pleuropneumoniae*  $\Delta$ *arcA* does not show  
37 autoaggregation under anaerobic conditions and is deficient in biofilm formation. It is  
38 hypothesised that the lack of these functions is, at least in part, responsible for the  
39 reduction of virulence.

40 **1. Introduction**

41

42 *Actinobacillus (A.) pleuropneumoniae* is the causative agent of a porcine  
43 pleuropneumonia causing high economic losses worldwide (Fenwick and Henry,  
44 1994). After inhalation of *A. pleuropneumoniae* containing aerosols the pathogen  
45 colonizes the respiratory epithelium of its host and persists on tonsils, on healthy lung  
46 epithelium, and in sequestered lesions (Sidibe et al., 1993). Our previous studies  
47 implied that the ability to adapt to anaerobic conditions is crucial for the development  
48 of persistent infections; thus, the deletion of *hlyX*, the homologue to the global  
49 regulator for anaerobic metabolism FNR in *E. coli* (Shaw and Guest, 1982), caused a  
50 clear attenuation of *A. pleuropneumoniae* (Baltes et al., 2005).

51 The ArcAB two-component system, like FNR, is a global regulatory  
52 mechanism facilitating the adaptation to a changing redox potential and anaerobic  
53 conditions in enterobacteria (Iuchi and Lin, 1988). The ArcAB system is composed of  
54 the transmembrane sensor kinase ArcB and the cytosolic response regulator ArcA  
55 (Iuchi and Lin, 1995, Lynch and Lin, 1996). *Salmonella arcA* mutants have been  
56 shown to grow more slowly than the wild-type strain in both aerobic and microaerobic  
57 conditions (Sevcik et al., 2001), and *E. coli arcA* mutants are unable to survive  
58 prolonged starvation (Nystrom et al., 1996). For *Haemophilus influenzae* deletion of  
59 *arcA* is accompanied with attenuation in septic infection (Souza-Hart et al., 2003),  
60 and in the intestinal pathogen *Vibrio cholerae* ArcA was found to modulate the  
61 expression of virulence factors (Sengupta et al., 2003).

62 The role of ArcA in an extracellular respiratory tract pathogen has not been  
63 investigated to date. Therefore, we constructed an isogenic *A. pleuropneumoniae*  
64 *arcA* deletion mutant, investigated its growth under aerobic and anaerobic conditions  
65 and determined its virulence in a pig aerosol infection model.

## 66 2. Materials and methods

67

### 68 2.1. Bacterial strains, plasmids, and primers

69

70 Bacterial strains, plasmids, and primers used in this work are listed in Table 1.

71

### 72 2.2. Media and growth conditions

73

74 *E. coli* strains were cultured in Luria-Bertani medium supplemented with the  
75 appropriate antibiotics (ampicillin, 100 µg/ml; chloramphenicol, 25 µg/ml) at 37°C; for  
76 cultivation of *E. coli* β2155 ( $\Delta dapA$ ), diaminopimelic acid (1 mM; Sigma Chemical  
77 Company, Deisenhofen, Germany) was added. *A. pleuropneumoniae* parent and  
78 mutant strain were cultured at 37°C and 5% CO<sub>2</sub> in PPLO medium or on PPLO agar  
79 (Difco GmbH, Augsburg, Germany) supplemented with NAD (10 µg/ml; Merck AG,  
80 Darmstadt, Germany), L-cysteine hydrochloride (260 µg/ml; Sigma), L-cysteine  
81 dihydrochloride (10 µg/ml; Sigma), and dextrose (1 mg/ml). The aerobic culture  
82 grown for experimental aerosol infection in addition contained Tween<sup>®</sup>80 (0.1%). For  
83 selection of *A. pleuropneumoniae* transconjugants (single crossing-overs)  
84 chloramphenicol (5 µg/ml) was added, and the medium for counter selection was  
85 prepared as described previously (Tonpitak et al., 2002). For cultivation of the  
86 complemented mutant kanamycin (25 µg/ml) was added.

87 For anaerobic growth supplemented medium was preincubated in an  
88 anaerobic chamber (DonWhitley Scientific, Shipley, England) in an atmosphere  
89 containing 5% CO<sub>2</sub>, 10% H<sub>2</sub>, and 85% N<sub>2</sub> at 37°C and then inoculated with 1% of log  
90 phase aerobic culture with an optical density at 600 nm (OD<sub>600</sub>) of 0.3. Anaerobicity  
91 of the medium was confirmed using a dissolved oxygen sensor (CellOx<sup>®</sup> 325; WTW,

92 Weilheim, Germany) linked to an inoLab<sup>®</sup> instrument (WTW, Weilheim, Germany).  
93 For recording of growth curves bacteria were harvested at 2, 4, 6, 8, 10 and 24 h  
94 post inoculation by centrifugation. As *A. pleuropneumoniae* showed heavy clumping  
95 upon growth under anaerobic conditions the protein content of whole-cell lysates was  
96 determined. Statistical analyses were performed using the Student's t-test.

97

### 98 *2.3. Preparation of whole-cell lysates*

99

100 Cells were harvested by centrifugation (13,000 x g, 10 min, 4°C), washed once  
101 with a buffer containing 10 mM Tris-HCl (pH 8.0) and 5 mM magnesium acetate.  
102 Pellets were resuspended in 50 mM Tris-HCl (pH 7.3) and stored at -70°C overnight.  
103 Cells were thawed, ruptured using the FastPrep<sup>®</sup> Instrument (Qbiogene, Heidelberg,  
104 Germany) three times for 40 sec at intensity setting 5.0, and cell debris was removed  
105 by centrifugation (15,000 x g, 30 min). Protein concentration was determined with the  
106 MicroBC<sup>®</sup> assay (Uptima Interchim, Montlucon Cedex, France).

107

### 108 *2.4. Manipulation of DNA*

109

110 DNA modifying enzymes were purchased from New England Biolabs (Bad  
111 Schwalbach, Germany) and used according to the manufacturer's instructions. Taq  
112 polymerase was purchased from Gibco-BR Life Technologies (Karlsruhe, Germany).  
113 Chromosomal and plasmid DNA preparations, PCR, Southern blotting,  
114 transformation, and gel electrophoresis were done by standard procedures  
115 (Sambrook et al., 1989), and pulsed field gel electrophoresis (PFGE) was performed  
116 as described previously (Oswald et al., 1999a).

117

118 2.5. Construction of an isogenic *A. pleuropneumoniae* *arcA* in-frame mutant

119

120 The upstream and downstream parts of the *arcA* gene and flanking sequence  
121 were amplified from genomic *A. pleuropneumoniae* AP76 DNA using primers oArcA1  
122 and oArcA2 or oArcA3 and oArcA4 and cloned into pCR2.1 TOPO, resulting in  
123 pARC810 and pARC800, respectively (Table 1). The downstream fragment was  
124 removed from pARC800 using HindIII and BsmBI and cloned into plasmid pARC810  
125 to obtain pARC820 harbouring the truncated *arcA* sequence. Plasmid pARC820 was  
126 restricted with NotI and PspOMI, and the 1264-bp fragment was ligated into the  
127 conjugation vector pEMOC2 resulting in plasmid pARC700. Plasmid pARC700 was  
128 used in the single step transconjugation system (Oswald et al., 1999b) to construct *A.*  
129 *pleuropneumoniae*  $\Delta$ *arcA*. Colonies with the correct PCR profile were confirmed by  
130 Southern blot analysis (Sambrook et al., 1989) using the PCR product obtained with  
131 primers oArcA5 and oArcA6 from *A. pleuropneumoniae*  $\Delta$ *arcA* as a probe, and by  
132 nucleotide sequence analysis (Seqlab, Göttingen, Germany). The absence of  
133 genomic rearrangements was confirmed by PFGE.

134

135 2.6. Complementation of *A. pleuropneumoniae*  $\Delta$ *arcA*

136

137 A 1236 bp fragment was obtained by PCR using *A. pleuropneumoniae* AP76  
138 genomic DNA and primers oArcA1 and oArcA8 (Table 1). This PCR product harbours  
139 505 bp of *arcA* upstream sequence likely including regulatory sequences crucial for  
140 *arcA* expression. The PCR products was cloned into pCR2.1 TOPO, restricted with  
141 EcoRI and ligated into EcoRI restricted plasmid pLS88 yielding pARC1340,  
142 containing the *arcA* gene in a 5'-3' orientation with respect to the vector-derived *sulll*  
143 promoter. The plasmid was electroporated into *A. pleuropneumoniae*  $\Delta$ *arcA*.



144 2.7. *Virulence studies*

145

146 Virulence of *A. pleuropneumoniae*  $\Delta$ *arcA* compared to the parental strain was  
147 assessed in an aerosol infection model as previously described (Maas et al., 2006).  
148 *A. pleuropneumoniae*-free pigs (no clinical symptoms and no serological responses  
149 in the ApxII-ELISA ([Leiner et al., 1999]), 8 to 9 weeks of age, were randomly  
150 assigned to the different groups (8 pigs per group) and cared for in accordance with  
151 the principles outlined by the European Convention for the Protection of Vertebrate  
152 Animals Used for Experimental and Other Scientific Purposes (European Treaty  
153 Series, no. 123 [<http://conventions.coe.int/treaty/EN/V3menutraitites.asp>; permit no.  
154 05/919]). Clinical examinations were performed daily or as needed. Body  
155 temperature and clinical symptoms were recorded as described before (Maas et al.,  
156 2006). Statistical analysis of the total clinical score was performed using the  
157 Wilcoxon Signed-Rank Test. Pigs were euthanized by intravenous injection of  
158 pentobarbital. Post mortem analysis and determination of reisolation score were  
159 performed as described previously (Maas et al., 2006).

160

161 2.8. *Autoaggregation, electron microscopy and biofilm assays*

162

163 *A. pleuropneumoniae* was grown in supplemented liquid medium under  
164 anaerobic conditions for six hours. Then cultures were removed from the anaerobic  
165 chamber and transferred on ice. For photography the liquid cultures were transferred  
166 into petri dishes.

167 To perform raster electron microscopy these anaerobically grown cultures  
168 were transferred onto 0.2  $\mu$ m membrane filters (Schleicher & Schuell, Dassel,

169 Germany) and fixed with 2 N dimethylarsinic acid and 25 % (w/v) glutaraldehyde.  
170 Electron microscopy was performed at 10,000 fold enlargement.

171 For the investigation of biofilm formation 20 ml of supplemented liquid medium  
172 in 200 ml glass Erlenmeyer flasks were inoculated with colony material from an agar  
173 plate and incubated for 6 h, 12 h, 18 h or 24 h at 37°C and 5% CO<sub>2</sub> without shaking.  
174 After incubation the OD<sub>600</sub> was measured. The bacterial suspension was removed  
175 and the flasks were washed rigorously with water before staining. Then 2 ml of a  
176 0.3% crystal violet solution (Merck) were added and the flasks were incubated for 5  
177 min with gentle agitation. After staining the flasks were washed rigorously with water  
178 and then dried. The crystal violet-stained biofilm on the glass surface was solubilized  
179 with 10 ml of a solution comprised of 50% ethanol, 30% acetone and 20% acetic  
180 acid. The OD<sub>600</sub> was determined and, using a calibration curve, the amount of crystal  
181 violet bound by the biofilm was calculated. All strains were tested in a minimum of  
182 four independent experiments. The flasks used were new and intensively cleaned  
183 and washed with distilled water between experiments. Statistical analyses were  
184 performed using the Student's t-test.

185

### 186 **3. Results**

187

#### 188 *3.1. Construction of an arcA deletion mutant of A. pleuropneumoniae*

189

190 A BLAST search identified a protein (gi:53729099) encoded on the genome of  
191 *A. pleuropneumoniae* strain 4074 which was 80 % identical to *E. coli* ArcA  
192 (gi:16132218) at the amino acid level. PCR and nucleotide sequence analysis  
193 revealed that a protein entirely identical to the *A. pleuropneumoniae* serotype 1 ArcA  
194 homologue is encoded by *A. pleuropneumoniae* AP76.

195 An isogenic *arcA* deletion mutant of *A. pleuropneumoniae* AP76 was  
196 constructed by allelic exchange of the wild type *arcA* gene with an in frame deletion  
197 lacking 186 bp at position 94 to 279 of the *arcA* ORF. The truncated ArcA protein  
198 lacks the putative phosphorylation site on Asp54. The resulting *A. pleuropneumoniae*  
199 *arcA* mutant was designated as *A. pleuropneumoniae*  $\Delta arcA$ ; it was verified using  
200 PCR, Southern blotting, and nucleotide sequence analysis; the absence of gross  
201 genomic rearrangements was confirmed by PFGE (supplementary material, Fig. S1).  
202 For complementation *A. pleuropneumoniae*  $\Delta arcA$  was transformed with plasmid  
203 pARC1340 and designated as *A. pleuropneumoniae*  $\Delta arcA$  + pARC1340.

204

### 205 3.2. Virulence studies

206

207 Aerosol infection with both *A. pleuropneumoniae* wt (group 1) and *A.*  
208 *pleuropneumoniae*  $\Delta arcA$  (group 2) led to an increase in body temperature above  
209 40°C in all infected animals. However, animals infected with *A. pleuropneumoniae*  
210  $\Delta arcA$  showed a significantly lower clinical score ( $p = 0.043$ ) than animals infected  
211 with *A. pleuropneumoniae* wt (Fig. 1A).

212 In order to investigate if the *arcA* deletion mutant could persist at least one  
213 week we decided to sacrifice four animals in group 2 seven days post infection. All  
214 remaining animals were sacrificed on day 21 post infection. At necropsy, three of  
215 eight pigs in group 2 had no macroscopically visible lung lesions compared to one  
216 out of eight animals in group 1. The lung lesion score of group 2 was clearly lower  
217 than that of group 1 (Fig. 1B). Histological examination revealed no differences in the  
218 morphology of lung lesions in both groups.

219 Reisolation of the challenge strain from intact lung at necropsy 21 days post  
220 infection succeeded in all animals of group 1 but only in one out of four animals of

221 group 2; at necropsy on day 7 post infection reisolation succeeded in two out of four  
222 animals of group 2 (Table 2). The reisolation score of *A. pleuropneumoniae*  $\Delta$ *arcA*  
223 was clearly reduced in comparison to the *A. pleuropneumoniae* wt group (Fig. 1C).  
224 From pneumonic lung the challenge strain could be reisolated in all seven animals  
225 with lesions in group 1 and in four out of five animals with lesions in group 2 with no  
226 difference seen between day 7 and day 21 post infection (Table 2).

227 Serum samples were obtained 1 week before and 3 weeks after experimental  
228 infection. Before infection, no *A. pleuropneumoniae* specific antibodies could be  
229 detected. On day 21 post challenge animals in both groups showed a strong humoral  
230 immune response, and no significant difference was observed (Table 2).

231

### 232 3.3. Growth and survival of *A. pleuropneumoniae* $\Delta$ *arcA*

233

234 The growth rates between *A. pleuropneumoniae* wt and *A.*  
235 *pleuropneumoniae*  $\Delta$ *arcA* exhibited no significant differences over a 24 h incubation  
236 period under both aerobic (Fig. S2 A) or anaerobic (Fig. S2 B) conditions. In addition,  
237 long term survival kinetics of *A. pleuropneumoniae* wt and *A. pleuropneumoniae*  
238  $\Delta$ *arcA* in an anaerobic liquid culture were indistinguishable. Sixty hours post  
239 inoculation no culturable *A. pleuropneumoniae* wt or *A. pleuropneumoniae*  $\Delta$ *arcA*  
240 were detectable (Fig. S2 C).

241

### 242 3.4. Effect of *arcA* deletion on bacterial autoaggregation and biofilm formation

243

244 In liquid culture under aerobic or microaerophilic conditions both *A.*  
245 *pleuropneumoniae* wt and *A. pleuropneumoniae*  $\Delta$ *arcA* grew as a homogenous

246 suspension (data not shown). Upon growth under anaerobic conditions *A.*  
247 *pleuropneumoniae* wt showed heavy autoaggregation. This phenotype was  
248 completely abolished by deletion of the *arcA* gene, and transformation of *A.*  
249 *pleuropneumoniae*  $\Delta$ *arcA* with plasmid pARC1340 restored the autoaggregating  
250 phenotype (Fig. 2A).

251 Raster electron microscopy of anaerobically grown cultures showed that *A.*  
252 *pleuropneumoniae* wt was embedded in a threadlike extracellular matrix. In *A.*  
253 *pleuropneumoniae*  $\Delta$ *arcA* an exogenous threadlike compound could also be  
254 observed. However, it seemed not to be attached to the bacterial surface, and  
255 threads had a relaxed structure. Complementation of *A. pleuropneumoniae*  $\Delta$ *arcA*  
256 with plasmid pARC1340 resulted in a phenotype which appeared to be associated  
257 even more tightly to the extracellular matrix than the wt strain (Fig. 2B).

258 As autoaggregation and the ability to form biofilm have been reported to be  
259 associated (Schembri et al., 2003) we investigated the ability of *A. pleuropneumoniae*  
260  $\Delta$ *arcA* to form biofilms. *A. pleuropneumoniae* wt attached to glass surfaces and  
261 formed biofilm. Six hours after inoculation was no significant difference in biofilm  
262 formation on the bottom of the Erlenmeyer flask between *A. pleuropneumoniae* wt  
263 and *A. pleuropneumoniae*  $\Delta$ *arcA*. In *A. pleuropneumoniae* wt the biofilm further  
264 increased until 12 hours post inoculation (p. i.). Only at 18 p. i. a reduction of biofilm  
265 mass obtained by quantitative crystal violet staining was apparent. In *A.*  
266 *pleuropneumoniae*  $\Delta$ *arcA*, however, the amount of biofilm was clearly reduced in  
267 comparison to the wildtype at 12 hours p. i., and the difference was statistically  
268 significant at 18 and 24 hours p.i. ( $p = 0.013$  and  $0.014$ , respectively, Fig. 3A).  
269 Complementation of *A. pleuropneumoniae*  $\Delta$ *arcA* in trans with plasmid pARC1340  
270 slightly overcompensated the defect in biofilm formation, which likely is due to the

271 multiple copy number of *arcA* in the pARC1340 transformants; transformants  
272 containing the empty plasmid vector pLS88 behaved like the wild type (Fig. 3B). The  
273 lack of biofilm formation in *A. pleuropneumoniae*  $\Delta arcA$  was not caused by  
274 differences in cell densities as obtained by determination of OD<sub>600</sub> (data not shown).

275

#### 276 **4. Discussion**

277

278 Anaerobic metabolism generally is a virulence-associated trait in the  
279 respiratory tract pathogen *A. pleuropneumoniae* (Baltes et al., 2003, Jacobsen et al.,  
280 2005, Baltes et al., 2005). Since bacteria are also able to sense low oxygen  
281 concentrations by the ArcAB system (Georgellis et al., 2001) and since ArcA-  
282 dependent regulation of virulence factors has been shown for the intestinal pathogen  
283 *Vibrio cholerae* (Sengupta et al., 2003) we set out to investigate if a comparative  
284 relationship between virulence and ArcA also occurs in a respiratory tract pathogen.

285 The finding that the *A. pleuropneumoniae*  $\Delta arcA$  strain constructed was highly  
286 attenuated initially led us to hypothesize that the physiological key features described  
287 for *arcA* mutants of *Enterobacteriaceae* (i.e. delayed growth under anaerobic [Sevcik  
288 et al., 2001] or under aerobic conditions [Oshima et al., 2002] or reduced long term  
289 survival [Nystrom et al., 1996]) might be responsible. However, contrary to its role in  
290 *Enterobacteriaceae* *A. pleuropneumoniae* ArcA did influence neither growth nor  
291 survival and, therefore, appears to have different functions in *A. pleuropneumoniae*.

292 We observed that deletion of ArcA reduced the ability of *A. pleuropneumoniae*  
293 for autoaggregation and biofilm formation under oxygen-deprived growth conditions,  
294 and both phenotypes are known to be associated (Schembri et al., 2003). Since  
295 biofilm formation has been shown to be common for field isolates of *A.*  
296 *pleuropneumoniae* (Kaplan and Mulks, 2005), we hypothesized that the attenuation

297 of *A. pleuropneumoniae*  $\Delta$ *arcA* might be linked to this defect in autoaggregation and  
298 biofilm formation. To investigate possible causes for this defect we performed  
299 scanning electron microscopy and observed a difference in threadlike extracellular  
300 material between *A. pleuropneumoniae* wt and *A. pleuropneumoniae*  $\Delta$ *arcA*. This  
301 extracellular matrix - although present – appears to not be appropriately anchored to  
302 *A. pleuropneumoniae*  $\Delta$ *arcA*. It might be PGA, a linear exopolysaccharide composed  
303 of N-acetylglucosamine which has been described for *A. pleuropneumoniae* to be  
304 involved in biofilm formation by acting as an extracellular adhesin (Kaplan et al.,  
305 2004; Izano et al., 2007). Alternatively, the structures might be extracellular DNA as  
306 suggested to be present in *Haemophilus influenzae* (Jurcisek and Bakaletz, 2007) or  
307 adhesive pili as shown in *Actinobacillus actinomycetomcomitans* (Fine et al., 1999).  
308 A reason for the apparent defect in association of cells and matrix in *A.*  
309 *pleuropneumoniae*  $\Delta$ *arcA* might be the lack of an ArcA regulated surface anchor or,  
310 alternatively, an upregulation of the biofilm releasing enzyme dispersin B (Kaplan et  
311 al., 2004) cutting the fibres of an extracellular N-acetylglucosamine polymer that  
312 binds to the bacterial surface. These results, which provide evidence for a direct  
313 influence of ArcA on biofilm formation, are supported by a recent report showing that  
314 the deletion of *arcA* in *E. coli* reduces competitiveness in biofilm formation (Junker et  
315 al., 2006).

316 Taken together our results support the hypothesis that ArcA of *A.*  
317 *pleuropneumoniae* is less important in acute infection but plays an essential role in  
318 respiratory tract persistence of *A. pleuropneumoniae*. This implies that an in-depth  
319 investigation of the ArcA regulon in the respiratory tract pathogen *A.*  
320 *pleuropneumoniae* might lead to the identification of previously unknown persistence-  
321 associated factors and metabolic pathways and, therefore, improve our

322 understanding of the mechanisms of host-pathogen interaction in chronic and latent  
323 infection.

324

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326

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331 Armgard Janczikowski from the Leibniz University Hannover.

332

### 333 **Appendix A. Supplementary data**

334

335 Fig. S1. Verification of *arcA* deletion by PCR, Southern blot analysis, and PFGE.

336 Fig. S2. Aerobical and anaerobical growth and anaerobical survival in liquid medium.



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- 437

438 Table 1

## 439 Bacterial strains and primers used in this study

Strain, plasmid or primer	Characteristic(s)	Source and/or reference
Strains		
<i>A. pleuropneumoniae</i> wt	<i>A. pleuropneumoniae</i> serotype 7 isolate 76	(Anderson et al., 1991)
<i>A. pleuropneumoniae</i> $\Delta$ <i>arcA</i>	Unmarked <i>arcA</i> -negative deletion mutant of <i>A. pleuropneumoniae</i> wt	This work
Plasmids		
pCR2.1-TOPO	<i>E. coli</i> cloning vector for fast and efficient cloning of <i>Taq</i> polymerase-amplified PCR products	TOPO TA cloning, Invitrogen, Groningen, The Netherlands (Shuman, 1994)
pARC800	pCR2.1-TOPO based plasmid containing a 683-bp PCR product obtained with primers oArcA3 and oArcA4, starting at position 276 of the <i>arcA</i> gene and ending 221 bp downstream of the <i>arcA</i> stop codon	This work
pARC810	pCR2.1-TOPO based plasmid containing a 622-bp PCR product obtained with primers oArcA1 and oArcA2, starting 505 bp upstream of the <i>arcA</i> start codon and ending at position 88 of the <i>arcA</i> gene	This work
pARC820	Plasmid obtained by digestion of pARC800 with EcoRI and BsmBI and ligation of the resulting 728-bp fragment into pARC810 digested with EcoRI and BsmBI	This work
pARC850	pCR2.1-TOPO based plasmid containing a 1236 bp PCR product obtained with primers oArcA1 and oArcA8, starting at position 505 bp upstream of the <i>arcA</i> start codon and ending at position 2 bp downstream the <i>arcA</i> stop codon	This work
pEMOC2	Transconjugation vector based on pBluescript SK with <i>mobRP4</i> , a polycloning site, Cm <sup>r</sup> , and transcriptional fusion of the <i>omIA</i> promoter with the <i>sacB</i> gene	Accession no. AJ868288 (Baltes et al., 2003)
pARC700	pEMOC2 based plasmid carrying the truncated <i>arcA</i> ORF excised from pARC820 using NotI and PspOMI and ligated into the pEMOC2 restricted with NotI and PspOMI	
pLS88	Broad-host-range shuttle vector from <i>Haemophilus ducreyi</i> ; Str <sup>r</sup> Sm <sup>r</sup> Km <sup>r</sup>	(Willson et al., 1989)
pARC1340	pLS88-based plasmid carrying the PCR product of primers oArcA1 and oArcA8 encoding the <i>arcA</i> ORF and 505 bp upstream sequence cloned into the EcoRI restriction site.	This work
Primers		
oArcA1	5' TAACGCGGCCGCCGCAAGAACGTATTTGGCG 3'; forward primer containing an internal NotI site (underlined) comprising positions 505-486 upstream of the <i>arcA</i> gene start codon	This work
oArcA2	5' AATCCGTCTCTCCAAACTTCATAACCTTCCGCCTCG 3'; reverse primer containing an internal BsmBI site (underlined) comprising positions 88-69 of the <i>arcA</i> gene	This work
oArcA3	5' GATCCGTCTCTTTGGAAATCGGTGCGGACG 3'; forward primer containing an internal BsmBI site (underlined) comprising positions 276-295 of the <i>arcA</i> gene	This work
oArcA4	5' ATCGGGGCCACGACTAACGCAGGACCGGT 3'; reverse primer containing an internal PspOMI site (underlined) comprising positions 202-221 downstream of the <i>arcA</i> gene stop codon	This work
oArcA5	5' CGCGCAATACGCTCAAAAGT 3'; forward primer comprising positions 44-63 of the <i>arcA</i> gene	This work
oArcA6	5' CCGGTGTATTAGGGTATCT 3'; reverse primer comprising position 660-641 of the <i>arcA</i> gene	This work
oArcA8	5' TACTACTCTAACTCGCCGCA 3'; reverse primer comprising position 2 downstream the <i>arcA</i> stop codon to position 700 of the <i>arcA</i> gene	This work

440 Table 2

441 Virulence of *A. pleuropneumoniae* parent and  $\Delta arcA$  mutant strain following aerosol

442 challenge

443

Challenge strain	No. of animals	Challenge dose (OD <sub>600</sub> [aerosolized] per 4 pigs) <sup>a</sup>	Necropsy time (day)	Serological response to:		No. of animals with reisolation of <i>A. pleuropneumoniae</i> at post mortem analysis in:			
				Detergent wash <sup>b</sup>	ApxIIA <sup>c</sup>	Tonsil	Lymph node	Lung	
								Pneumonic	Intact
AP76 wt	8	0.47	21	3275 ± 2742	28.9±23.6	4/8	6/8	7/7	8/8
AP76 $\Delta arcA$	4	0.42	7	-	-	4/4	2/4	2/2	2/4
AP76 $\Delta arcA$	4		21	3000 ± 2477	60.5±25.7	1/4	2/4	2/3	1/4

444

445 <sup>a</sup> Bacteria were grown aerobically in the presence of Tween<sup>®</sup>80 (0.1%) to the  
 446 respective OD<sub>600</sub> and diluted 1:30,000 with sterile saline; 13 ml of this dilution were  
 447 aerosolized in the aerosol chamber.

448 <sup>b</sup> The solid-phase antigen was prepared as described previously (Goethe et al.,  
 449 2000); the number given is the arithmetic mean of the highest serum dilution  
 450 resulting in an optical density twice as high as the negative control serum at a dilution  
 451 of 1:100

452 <sup>c</sup> Recombinant ApxIIA protein was used as solid-phase antigen as described  
 453 previously (Leiner et al., 1999); the number given is the arithmetic mean of the  
 454 serum activity in ELISA units.

455 **Figure legends**

456

457 Fig 1. Virulence of *A. pleuropneumoniae* wt and *A. pleuropneumoniae*  $\Delta$ *arcA*  
458 compared in an aerosol infection model. Clinical score (A), lung lesion score (B) and  
459 reisolation score (C) of pigs infected with *A. pleuropneumoniae* wt (▲) or  $\Delta$ *arcA* (○  
460 [sacrificed on day 7]; ●[sacrificed on day 21]) . Each triangle or circle represents one  
461 animal. The horizontal line represents the arithmetic mean and the error bar denotes  
462 the standard deviation. The asterisks denote statistical significance ( $p < 0.05$ ) of  
463 differences between the entire *A. pleuropneumoniae*  $\Delta$ *arcA* group (animals sacrificed  
464 at day 7 [four animals] and 21 post infection [four animals]) and *A. pleuropneumoniae*  
465 wt group (all sacrificed on day 21 post infection) as obtained using the Wilcoxon  
466 Signed-Rank Test. The animals sacrificed at different time points post infection were  
467 considered as a single group since both lung lesion and reisolation scores do not  
468 increase after day 7 of an *A. pleuropneumoniae* infection. Therefore, the approach  
469 can have resulted at the most in an underestimation of differences between the two  
470 groups.

471

472 Fig. 2. Bacterial autoaggregation in anaerobic broth cultures (A, B) of *A.*  
473 *pleuropneumoniae* wt, *A. pleuropneumoniae*  $\Delta$ *arcA* and *A. pleuropneumoniae*  $\Delta$ *arcA*  
474 supplemented in trans with pARC1340. A; autoaggregation of anaerobically grown  
475 cultures. B; raster electron microscopy of cultures shown in A.

476

477 Fig. 3. Biofilm formation on the bottom of glass Erlenmeyer flasks. Comparison of *A.*  
478 *pleuropneumoniae* wt and *A. pleuropneumoniae*  $\Delta$ *arcA* (A) as well as of *A.*  
479 *pleuropneumoniae*  $\Delta$ *arcA* supplemented in trans with pARC1340 and *A.*



480 *pleuropneumoniae*  $\Delta$ *arcA* complemented with the empty vector pLS88 (B). The  
481 biofilm was stained with crystal violet. Crystal violet bound to the biofilm was  
482 solubilized and measured at OD<sub>600</sub>. Using a calibration curve the amount of crystal  
483 violet was calculated as ppm [w/v]. Error bars represent the standard deviation.  
484 Statistical analysis was performed using Student's t-test. Asterisks denote statistical  
485 significance ( $p < 0.05$ ).

1 **Legends to figures S1 to S2**

2

3 Fig. S1. Verification of *arcA* deletion by PCR, Southern blot analysis, and PFGE. A;  
4 Agarose gel electrophoresis of a DNA marker (Lambda phage restricted with HindIII  
5 (lane1) and PCR products obtained with primers oArcA5 and oArcA6 from genomic  
6 *A. pleuropneumoniae* DNA. PCR products obtained from *A. pleuropneumoniae* wt  
7 (lane 2) and *A. pleuropneumoniae*  $\Delta arcA$  (lane 3) were 618 bp and 432 bp  
8 respectively. B; Southern blot analysis of genomic DNA restricted with HaeIII and  
9 XmaI. Digestion of *A. pleuropneumoniae* wt DNA resulted in a 915 bp fragment (lane  
10 1), and a 729 bp fragment was obtained for *A. pleuropneumoniae*  $\Delta arcA$  (lane 2). C;  
11 PFGE analysis to exclude genomic rearrangements. Genomic DNA of *A.*  
12 *pleuropneumoniae* wt (lanes 1, 3, 5) and *A. pleuropneumoniae*  $\Delta arcA$  (lanes 2, 4, 6)  
13 was restricted with PspOMI (lanes 1, 2), Ascl (lanes 3, 4) and NotI (lanes 5, 6).  
14 Phage lambda concanamers were loaded as a size marker (lane 7).

15

16 Fig. S2. Analysis of anaerobic growth (A), aerobic growth (B) and survival under  
17 anaerobic conditions accompanied by starvation (C) of *A. pleuropneumoniae* wt and  
18 *A. pleuropneumoniae*  $\Delta arcA$ . Each experiment was repeated at least three times.  
19 Error bars represent the standard deviation.

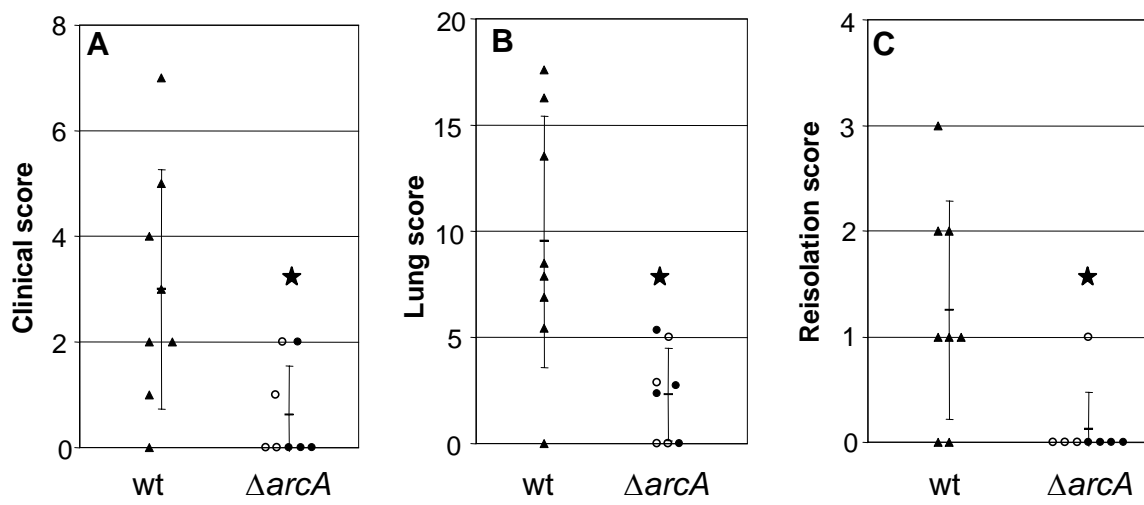
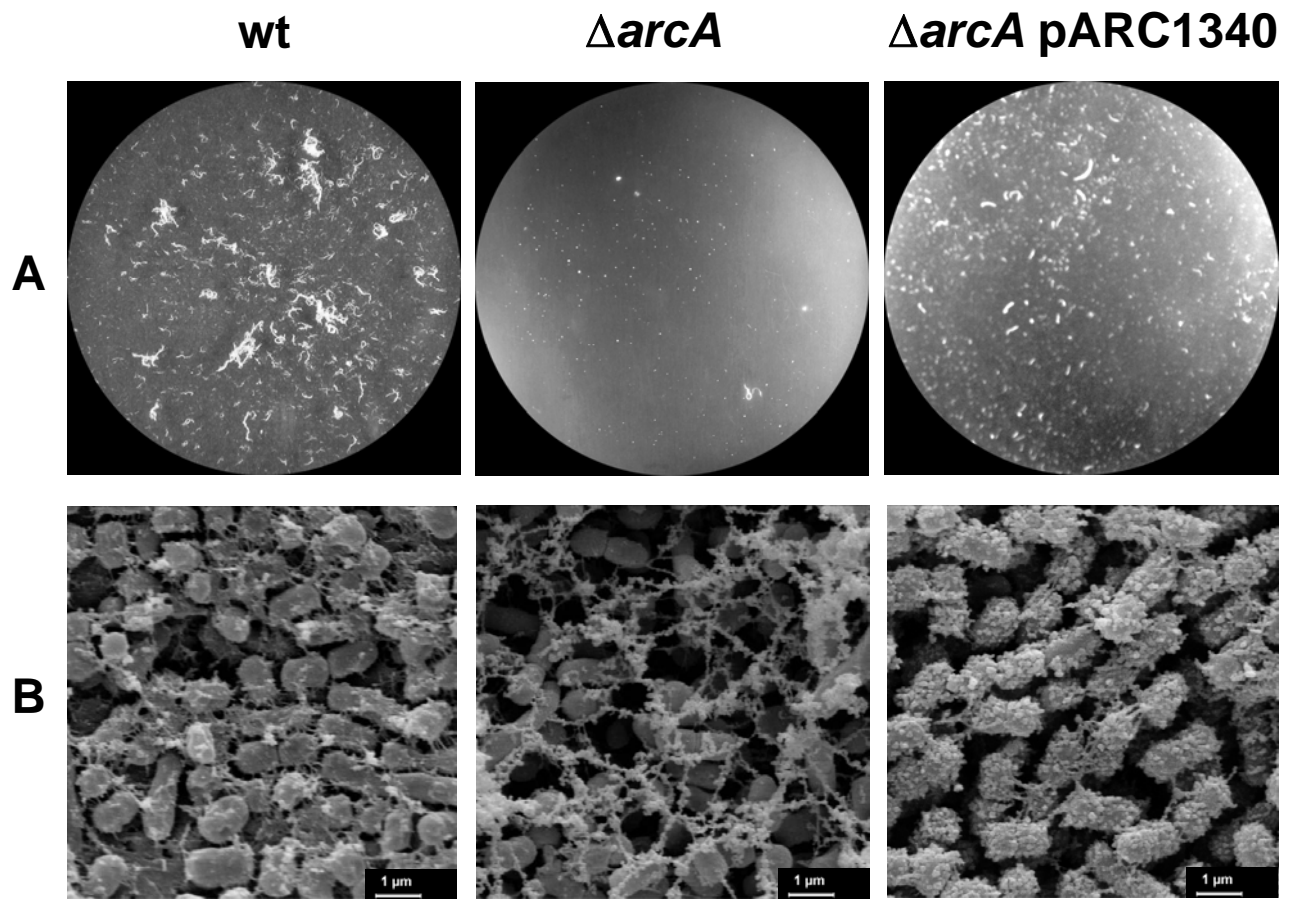


FIGURE 1

**FIGURE 2**

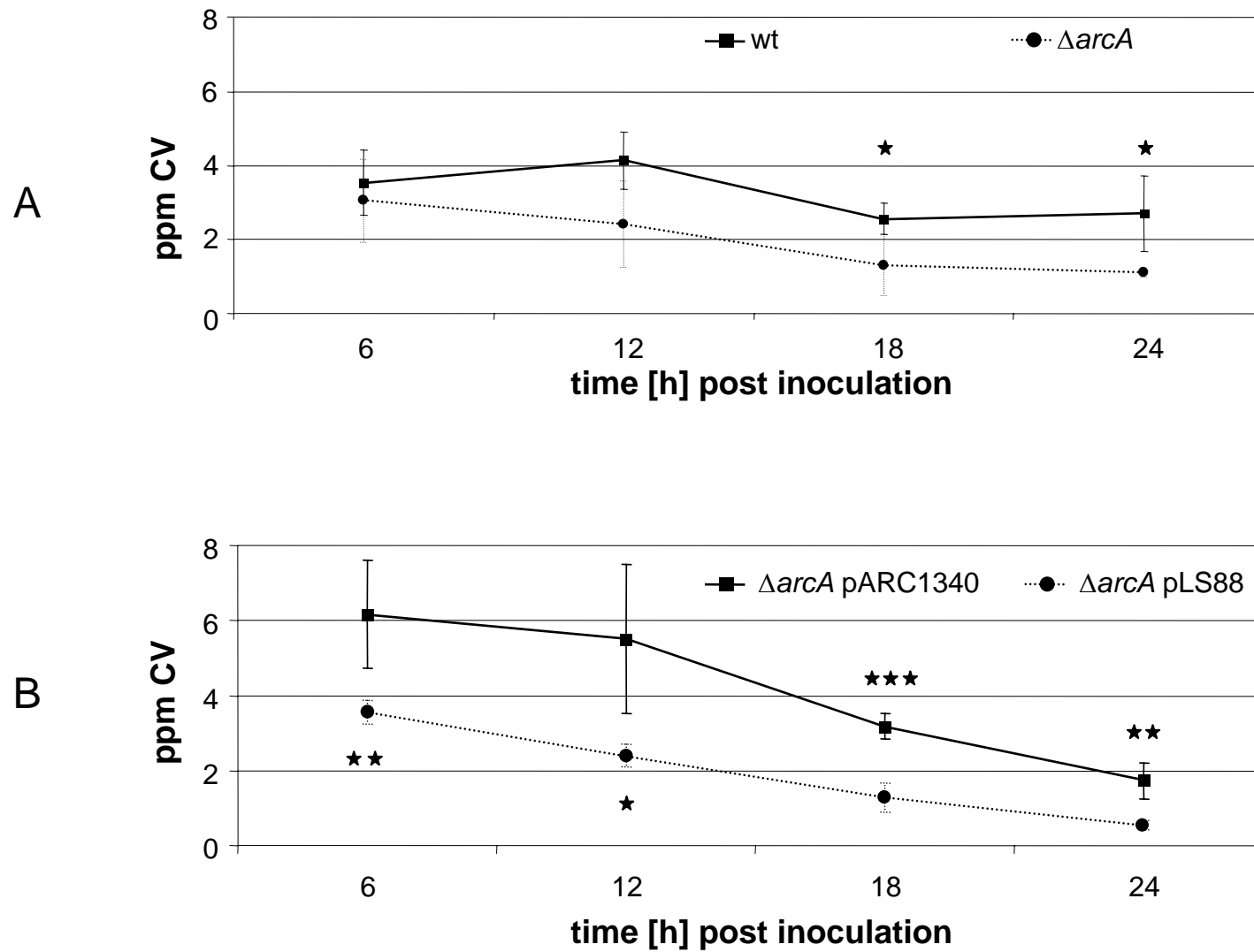


FIGURE 3