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**Gene regulation in *Actinobacillus pleuropneumoniae* -
Different approaches to determine the response to
iron limitation and anaerobiosis in vivo**

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"X" never, ever marks the spot.

Indiana Jones and the last Crusade (1989)

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List of abbreviations

®	registered trademark
μ	micro
A. bidest.	Aqua bidestillata
A. dest.	Aqua destillata
<i>A. pleuropneumoniae</i>	<i>Actinobacillus pleuropneumoniae</i>
AspA	aspartate ammonia lyase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bla	beta-lactam antibiotics resistance determinant
bp	base pair(s)
Bq	Becquerel
cDNA	complementary DNA
cfu	colony forming unit
Cm ^r	chloramphenicol resistance determinant
CSB	Columbia sheep blood
Da	Dalton
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
Erm ^r	erythromycin resistance determinant
et al.	et alii
FACS	fluorescent activated cell sorting
Fig.	figure

FP	fluorescent protein
Fur	Ferric uptake regulator
g	gram(s)
g	g force
GFP	green fluorescent protein
GST	glutathione-S-transferase
h	hour(s)
<i>H. ducreyi</i>	<i>Haemophilus ducreyi</i>
IPG	immobilized pH gradient
IPTG	isopropyl β -D-1-thiogalactopyranoside
k	kilo
Km ^r	kanamycin resistance determinant
kb	kilo base pair(s)
kDa	kilo Dalton
l	liter
LB	Luria Bertani
log	logarithmic
LPS	lipopolysaccharides
m	milli
M	molar
min	minute(s)
ml	milliliter
<i>M. bovis</i>	<i>Moraxella bovis</i>
n	nano
Na ₃ CaDTPA	diethylenetriamine-pentaacetic acid calcium trisodium salt hydrate
NaCl	sodium chloride
NAD	nicotine amide adenine dinucleotide
NBT	nitroblue tetrazolium
<i>N. gonorrhoeae</i>	<i>Neisseria gonorrhoeae</i>
<i>N. meningitidis</i>	<i>Neisseria meningitidis</i>
OD _{xxx}	optical density at xxx nanometers

OMP	outer membrane protein
ORF	open reading frame
p	pico
PCR	polymerase chain reaction
p. i.	post infectionem
<i>P. multocida</i>	<i>Pasteurella multocida</i>
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PMSF	phenylmethylsulfonyl fluoride
rfu	relative fluorescent unit
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rounds per minute
RT	room temperature
RT PCR	Reverse Transcriptase PCR
<i>S. Typhimurium</i>	<i>Salmonella Typhimurium</i>
SCLB	standard cell lysis buffer
SDS PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulphate
sec	second(s)
Str ^r	Streptomycin resistance determinant
TCA	trichloroacetic acid
™	trademark
Tbp	transferrin binding protein
U	unit
V	volt
v/v	volume by volume
W	watt
w/v	weight by volume
wt	wild type

1 Introduction

Actinobacillus (A.) pleuropneumoniae is a facultative anaerobic gram-negative rod belonging to the family of *Pasteurellaceae*. The bacterium is responsible for severe economic losses in the pig fattening industry because it is the causative agent of Porcine Pleuropneumonia, a highly contagious disease with a clinical appearance ranging from peracute death over acute pleuropneumonia with necrotic and hemorrhagic lesions to a symptom-free carrier status of the animals.

In its natural niche, the porcine respiratory tract, *A. pleuropneumoniae* has to adapt to an environment which is deprived of oxygen and iron. It has been shown recently that anaerobiosis significantly influences the protein expression of *A. pleuropneumoniae*. The two important anaerobic regulators ArcA and HlyX play a major role in these regulatory processes (BUETTNER 2008). Another task the bacterium encounters is to overcome the iron limitation in the lung. Factors facilitating the acquisition of iron, such as transferrin-binding proteins (Tbp) and the iron-regulated outer membrane protein B (FrpB), have been identified to be virulence-associated.

In order to further elucidate the molecular mechanisms involved, three tasks were to be performed in this thesis:

1. A GFP-labelled *A. pleuropneumoniae* was to be constructed, and it was to be investigated whether this “knock-in” mutant was stable and still virulent and whether GFP-expression was sufficiently strong to investigate gene regulation in vivo.
2. An *A. pleuropneumoniae* deletion mutant lacking the FrpB-encoding gene had been found to be avirulent (BENDALLAH 2008). The molecular cause for these results was to be investigated.
3. The protein Ape51 (annotated as “hypothetical”) was found to be immunogenic in a Western blot analysis with serum of pigs convalescent from *A. pleuropneumoniae* infection. The protein was predicted to be surface-associated and a homology search revealed a possible role in adherence. Further, the *ape51* gene is located directly downstream of the *arcA* gene encoding the global anaerobic regulator ArcA. Therefore it was to be investigated whether Ape51 was involved in regulation during anaerobiosis and whether it had an impact on virulence.

2 Literature review

2.1 *Actinobacillus pleuropneumoniae*

2.1.1 Taxonomy

A. pleuropneumoniae is a gram-negative bacterium belonging to the family of *Pasteurellaceae*. After it was originally classified as *Haemophilus pleuropneumoniae* (SHOPE et al. 1964), a high homology on the DNA level to *A. lignieresii* promoted the transfer to the genus *Actinobacillus* (POHL et al. 1983). Depending on their ability to synthesise nicotinic amid adenine dinucleotide (NAD), two biotypes can be distinguished. Biotype one is NAD dependent whereas biotype two can synthesise NAD independently (NICOLET 1992). According to discrimination by their surface polysaccharides, biotype 1 can be divided into 13 serotypes. Serotype 1 and 5 are subdivided in a and b, respectively, due to minor differences in the polysaccharide structure (ALTMAN et al. 1990; JOLIE et al. 1994; PERRY 1990). For biotype 2, six serotypes (NICOLET 1992; NIELSEN et al. 1997) are known to date.

2.1.2 Impact and epidemiology

A. pleuropneumoniae is the causative agent of Porcine Pleuropneumonia, a disease causing significant economic losses in pork production. Economic losses originate from poor growth rates of convalescent and chronically infected animals as well as from fatalities during the acute phase of disease (FENWICK and HENRY 1994). The disease was first described in the United Kingdom in 1957 by PATTISON et al. (1957) but today it is occurring worldwide with different serotypes being more prevalent in certain geographical areas. In Europe serotypes 2, 3, 6, 7, 8 and 9 can be found predominantly whereas there is a dominance of serotypes 1 and 5 in North America. On the Australian continent serotype 15 is the most common (BLACKALL et al. 2002; BLAHA 1992; CHIERS et al. 2002; FENWICK and HENRY 1994; SATRAN and NEDBALCOVA 2002).

The tenacity of *A. pleuropneumoniae* in the environment is very low and, in general, disinfection arrangements are highly effective. Transmission via live or dead vectors

is of minor importance; the common route of infection is the introduction of subclinically infected animals into an *A. pleuropneumoniae*-free herd (FENWICK and HENRY 1994). Therefore, a strict all-in and all-out policy should be followed instead of continuous restocking of animals. Additional factors contributing to the dissemination of the disease are poor hygiene and ventilation as well as crowding of the animals.

2.1.3 Infection, disease and immunology

A. pleuropneumoniae is highly host specific for pigs. However, isolation of the pathogen from lambs has been described (DSILVA et al. 1995; HERVAS et al. 1996). The spread of disease can be airborne but, more commonly, droplet infections over short distances and infections by direct animal-to-animal contact occurs (NICOLET et al. 1969; TAYLOR 1995; TORREMORELL et al. 1997). The course of disease ranges from peracute death over an acute respiratory disease to chronic infection. In addition, latently infected and convalescent carrier animals are observed. During the peracute form pigs present themselves with high fever, apathy and anorexia. Respiratory symptoms only occur in the terminal phase of peracute disease, and animals die of a cardiac and circulatory failure. Acute disease is characterised by fever, depression and severe respiratory symptoms with dyspnoea, coughing and vomiting; the chronic form develops in animals surviving acute disease. Weight gain is reduced, and spontaneous or intermittent cough can be observed. Pathological alterations consist of a fibrinous, necrotising haemorrhagic pneumonia with affection of the pericardium and possibly the joints. Lung alterations with the formation of sequesters and pleural lesions occur in the chronic phase of disease (NICOLET 1992). The severity of disease is dependent on the involved serotype, the infection dose and the immune status of the host (FENWICK and HENRY 1994).

In general, pigs of all age groups are susceptible to *A. pleuropneumoniae* infection. Studies by CHIERS et al. (2002) have shown that neutralising maternal antibodies can be found in piglets up to an age of 4 weeks. A gap of 12 weeks follows before seroconversion occurs in animals at an age of at least 16 weeks underlining why animals 10 to 16 weeks of age are most likely to contract acute disease.

2.2 Virulence and virulence associated-factors

Virulence is defined as the capability of a pathogen to cause disease. Bacterial products enhancing bacterial growth and survival in the host, thereby contributing to infection and disease, are defined as virulence factors (MAHAN et al. 1996; MEKALANOS 1992). Factors playing a role in virulence but also in bacterial metabolism are designated as virulence-associated factors. Important virulence and virulence-associated factors relevant for this study are reviewed below.

2.2.1 The role of fimbriae, outer membrane proteins, LPS and capsule in adherence

Adherence of *A. pleuropneumoniae* to the host's epithelial surfaces is not very well investigated. It has been described by VAN et al. (2002) that depletion of NAD in vitro, mimicking the in vivo situation, leads to an increase in adherence to porcine alveolar epithelial cells that might be connected with upregulation of fimbriae and outer membrane proteins.

Bacterial fimbriae are mediating adherence to the host's surface, the first step in colonisation; *A. pleuropneumoniae* fimbriae have been characterised as type 4 fimbriae. The promoter driving the genes encoding the fimbriae is activated upon host cell contact (BOEKEMA et al. 2004; STEVENSON et al. 2003; ZHANG et al. 2000).

Outer membrane proteins (OMPs) also seem to play a role in *A. pleuropneumoniae* adherence. Thus, several proteins have been described to be upregulated in conditions which positively influence adherence. These OMPs are of different molecular masses and, to our knowledge, their molecular identity has not been determined to date (VAN et al. 2002; HAMER-BARRERA et al. 2004).

BELANGER et al. (1990) have proposed that LPS plays a major role in adherence. In primary cell cultures of lung epithelial cells it has been described that adherence of *A. pleuropneumoniae* is masked by the O-antigen chain of LPS, as O-antigen-deficient mutants adhere better than their parent strains (BOEKEMA et al. 2003).

These findings supported the results of RIOUX et al. (1999) who found that the O-antigen is not necessarily involved in adhesion but the core lipid A region has to remain intact for adherence of *A. pleuropneumoniae*. In addition, PARADIS et al. (1994) found that the polysaccharides and not lipid A are responsible for adherence. Another factor involved in adherence is the bacterial capsule. The *A. pleuropneumoniae* capsule consists of non-branching polysaccharides and is non toxic. It inhibits phagocytosis and mediates serum resistance (FENWICK and OSBURN 1986; INZANA 1987). A distinct and adherent capsule is associated with a higher virulence and vice versa (JENSEN and BERTRAM 1986; ROSENDAL and MACINNES 1990). These results are contradicted by the findings of RIOUX et al. (2000) who showed that the capsule defends the bacterium against the host's immune system but that it is hindering adherence as non-capsulated mutant strains adhere better to frozen sections of piglet tracheal rings than the parent strain.

2.2.2 RTX toxins

The four Apx toxins of *A. pleuropneumoniae* belong to the family of RTX toxins (**R**epeats in **T**oxin) which are present in many gram-negative bacteria (WELCH 1991) and are secreted by type I secretion systems. They cause pores in the host cell membranes leading to cytolysis. In addition to the direct cell damage, the toxins trigger the release of inflammatory cytokines which in turn results in severe tissue damage. The toxins vary in distribution between the different serotypes of *A. pleuropneumoniae* and in biologic activity; thus, ApxI can be found in serotypes 1, 5, 9, 10 and 11 and is strongly haemolytic and cytotoxic for phagocytic cells. ApxII is expressed by all serotypes except serotypes 10 and 14 and is mildly haemolytic and cytotoxic. ApxIII, present in serotypes 2, 3, 4, 6, 8 and 15, is non-haemolytic but strongly cytotoxic towards alveolar macrophages and neutrophils (FREY et al. 1993). The ApxIV toxin is weakly haemolytic and present in all reference strains but it is expressed only in vivo. Recently TEGETMEYER et al. (2008) have identified an insertion element interrupting the *apxIV* open reading frame (ORF) of serotype 7 clinical isolate AP76, thereby preventing ApxIV expression and leading to a negative reaction of infected pigs in an ApxIV ELISA.

ApXI and ApXIII have been shown to significantly contribute to the severity of disease as animals endobronchially inoculated with culture supernatant or recombinant toxin developed severe clinical disease and lung lesions indistinguishable from lesions of pigs infected with the complete bacterium (KAMP et al. 1997). Furthermore *A. pleuropneumoniae* mutant strains lacking toxins I to III were avirulent in an infection study (INZANA et al. 1991; KAMP et al. 1997; PRIDEAUX et al. 1999). Recombinant ApXII toxin only induces mild clinical symptoms and few lesions when administered to pigs. The role of ApXIV in pathogenesis remains to be elucidated as it can be detected in phagocytic cells in the lung but its contributing effect to infection remains unclear (CHO and CHAE 2001; SCHALLER et al. 1999). However, as it is only expressed in vivo an ELISA with ApXIV as solid-phase antigen can be used to differentiate vaccinated from infected animals (DREYFUS et al. 2004).

2.2.3 Anaerobic respiration

The natural niche of *A. pleuropneumoniae* is the porcine respiratory tract, an environment characterised by very good aeration and blood perfusion. Nevertheless, major regulatory processes of *A. pleuropneumoniae* have been shown to be driven by anaerobiosis. During the course of infection severe tissue destruction with the formation of purulent encapsulated sequesters is evolving. These sequesters are characterised by poor blood and oxygen supply due to separation from the airways and the blood vessel system. Furthermore, glutathione, a reducing agent, is consistently present in epithelial lining fluid, increasing in concentration as a response to infection. These findings led to the hypothesis that anaerobic respiration induced by low redox potential and decreased oxygen tension might also play a role during the acute phase of disease (BALTES et al. 2005). Based on this hypothesis, an *A. pleuropneumoniae* deletion mutant lacking the DmsA subunit of the dimethyl sulfoxide (DMSO) reductase was constructed and shown to be attenuated in an animal experiment. It was hypothesised that its substrates function as alternative electron acceptors when oxygen is scarce (BALTES et al. 2003a).

Furthermore, the aspartate ammonia lyase (AspA) of *A. pleuropneumoniae* was found to be upregulated under anaerobic conditions. The activity of this enzyme

indirectly contributes to the formation of fumarate and thereby potentially enables fumarate respiration. Subsequently, an *A. pleuropneumoniae* *aspA dmsA* double mutant was constructed and found to be impaired in its ability to colonise and persist on the intact porcine respiratory tract epithelium thus impairing long term survival in the host (JACOBSEN et al. 2005b). These findings led to the conclusion that anaerobic metabolism plays a significant role in the pathogenesis of *A. pleuropneumoniae*.

2.2.4 Regulators involved in anaerobiosis

2.2.4.1 The *A. pleuropneumoniae* FNR homologue HlyX

The *A. pleuropneumoniae* HlyX protein has been identified as the homologue of the *Escherichia (E.) coli* Fumarate and Nitrate Reduction protein (FNR) (MACINNES et al. 1990); *E. coli* deletion mutants lacking the protein are not able to grow under anaerobic conditions because they can not perform fumarate and nitrate reduction (LAMB DEN and GUEST 1976) and, in *E. coli*, fumarate and nitrate also serve as terminal electron acceptors in anaerobic respiration. It was shown that FNR is an important regulator influencing more than 120 genes under anaerobic conditions (SAWERS et al. 1988). Complementation of *E. coli* Δ *fnr* with the *A. pleuropneumoniae* *hlyX* gene led to a haemolytic phenotype which was able to grow under anaerobic conditions. The ability of HlyX to induce a latent haemolysin in *E. coli* led to the conclusion that the regulons of these proteins are overlapping but not identical (GREEN and BALDWIN 1997).

An *A. pleuropneumoniae* Δ *hlyX* mutant was reduced in colonisation and persistence in an in vivo infection model although it was not found to be completely avirulent. This finding led to the hypothesis that *hlyX* deletion can, at least partly, be compensated by other regulators (BALTES et al. 2005). Global expression profiling of the *A. pleuropneumoniae* HlyX regulon revealed, like in *E. coli*, the regulation of a wide variety of proteins belonging to various functional classes. Like in *E. coli*, several terminal reductases transferring electrons to acceptors other than oxygen were strongly upregulated; among them is the above mentioned DMSO reductase underlining previous findings (BUETTNER 2008).

2.2.4.2 The global anaerobic regulator ArcA

The Anoxic redox control protein A (ArcA) of *A. pleuropneumoniae* belongs to the ArcAB two component system. ArcB is a transmembrane histidine sensor kinase connected to the cytosolic response regulator ArcA. The system enables bacteria to sense reducing conditions and to adapt gene expression accordingly (LYNCH and LIN 1996). The deletion of the *arcA* gene in *A. pleuropneumoniae* led to a significant attenuation in an animal experiment which is, amongst others, due to a reduced growth rate in vivo. Furthermore, the ability for formation of biofilms on abiotic surfaces was impaired in the ArcA mutant. It was concluded that *A. pleuropneumoniae* ArcA plays an essential role in long term persistence in the respiratory tract (BUETTNER et al. 2008b). In addition, expression profiling of the ArcA regulon underlined the relevance of ArcA for anaerobic respiration. This result was supported by proteomic analyses where it could be shown that ArcA modulates protein expression such that fumarate respiration is favoured. Thus, fumarate respiration seems to be involved in survival under anaerobic conditions and to contribute to virulence of *A. pleuropneumoniae* (BUETTNER et al. 2008a).

2.2.5 Iron acquisition

The limitation of iron in the host, in this case in the porcine lung, is a major defence mechanism against bacterial infection as iron is an essential bacterial nutrient. To overcome this limitation *A. pleuropneumoniae* has developed several iron uptake mechanisms enabling it to utilise iron from the host's haemin, haemoglobin (ARCHAMBAULT et al. 2003; BELANGER et al. 1995), and transferrin (ARCHAMBAULT et al. 2003; GERLACH et al. 1992; GONZALEZ et al. 1995; WILKE et al. 1997). Furthermore, different exogenous microbial siderophores can be used as iron sources (DIARRA et al. 1996). All mechanisms are requiring specific receptor proteins. The most potent ones for *A. pleuropneumoniae* are the transferrin binding proteins TbpB and TbpA. TbpB is characterised as an outer membrane-associated lipoprotein with a size of 60 kDa, and TbpA is an integral outer membrane protein with a size of 100 kDa. It has been shown by BALTES et al. (2002) that both *tbpB*

and *tbpA* deletion mutants cannot colonise the porcine respiratory tract and, therefore, are rendered avirulent.

In conformity with other bacteria, iron uptake is regulated by the ferric uptake regulator (Fur) (BAGG and NEILANDS 1987). Fur forms a complex with ferrous iron and in its iron-loaded form, acts as a transcriptional repressor. In an *A. pleuropneumoniae fur* deletion mutant the transferrin binding proteins are consistently expressed and the mutant is unable to cause clinical disease (JACOBSEN et al. 2005a).

2.2.6 Other factors

Urease catalyses the conversion of urea into ammonia and carbon dioxide thereby increasing the pH value of the surroundings. This reaction serves the bacterium in two ways: *A. pleuropneumoniae* can use the resulting ammonia as nitrogen source, and it balances the reduced pH value in inflamed tissue creating a more favourable environment. Although a spontaneous urease negative mutant has been isolated from a case of acute Porcine Pleuropneumonia, *A. pleuropneumoniae* field strains are usually urease positive implying a relevance of urease for virulence of the bacterium (BLANCHARD et al. 1993). These observations from the field are underlined by low-dose infection experiments with urease negative mutants which were not able to establish disease (BALTES et al. 2001; BOSSE and MACINNES 2000).

Under in vivo conditions *A. pleuropneumoniae* secretes proteases cleaving IgA thereby facilitating colonisation of the lower respiratory tract (KILIAN et al. 1979; NEGRETE-ABASCAL et al. 1994).

Protection from free oxygen radicals was shown to be provided by a Co-Zn-superoxide dismutase in vitro; however, no influence on virulence for this enzyme could be detected under in vivo conditions (LANGFORD et al. 1996; SHEEHAN et al. 2000).

The formation of biofilms enables bacterial persistence in environments planctonic bacteria would not be able to survive in. As the ability to form biofilms is prevalent in

most field isolates of *A. pleuropneumoniae*, it is considered a virulence-associated feature of the bacterium (KAPLAN and MULKS 2005).

Several other genes possibly associated with virulence could be identified in a signature-tagged mutagenesis approach by SHEEHAN et al. (2003). Their actual contribution, however, remains to be elucidated.

2.3 Fluorescent proteins

Fluorescent proteins (FPs) are a widely used tool for in vivo applications. The green fluorescent protein (GFP) discovered in the jellyfish *Aequorea victoria* was the first used as a marker for gene expression in 1994 (CHALFIE et al. 1994). It has advantages over chemical dyes and other bioluminescent markers, such as luciferase, as there is no need for an external substrate. Although the expression of fluorescent proteins is easy to achieve, it has to be kept in mind that a minimal amount of oxygen is required for correct folding of the protein. A crucial factor for selection of a suitable FP is its brightness which is required to achieve an adequate contrast and signal to noise-ratio (OLENYCH et al. 2007). Brightness is determined by the product of the molar extinction coefficient at the peak of the absorption band and the integrated emission quantum yield (RIZZO and PISTON 2005). Maturation of the proteins usually occurs at 37 °C.

The original wt GFP was genetically optimised resulting in the bright and photostable prototype for GFPs emitting in the spectral region of 500 to 525 nm which was designated as enhanced GFP (EGFP) (HEIM et al. 1995). The fluorescent protein used in this study is the GFPmut3 which has been optimised for use in fluorescent activated cell sorting (FACS). This mutant has a 21-fold higher fluorescence intensity than the wt when excited at a wavelength of 488 nm which is commonly used in FACS applications. This effect is partly due to a better solubility because of more efficient folding of the protein and to a shift in the absorption peak from 395 nm to 480 nm. The emission wavelength remains unchanged at 507 to 511 nm. A plasmid-based expression in *E. coli*, *Salmonella* (S.) Typhimurium and *Yersinia pseudotuberculosis* showed no species-specific differences in expression and fluorescence intensity of GFPmut3 (CORMACK et al. 1996).

A GFPmut3-labelled *S. Typhimurium* strain has successfully been used to orally infect mice. In a plasmid-based system the *gfp* gene was cloned in front of well-characterised promoters activated during infection, meaning that fluorescence only occurs when the bacterium is exposed to the respective conditions. As autofluorescence from the gut tissue hindered FACS-sorting of fluorescent *Salmonella*, detection was possible only when a certain threshold in fluorescence intensity was reached (BUMANN 2002). Thus, as not all bacteria express GFP above the threshold, it is not possible to quantify the bacterial load. A second problem is that plasmid-based expression of GFP at high levels (approximately more than 200,000 copies per cell) interferes with bacterial growth thereby altering the course of infection (WENDLAND and BUMANN 2002). A solution to these problems might be the insertion of a single-copy *gfp* fusion into the bacterial chromosome. This would limit the copy number of GFP in the cell and facilitate quantification due to improved homogeneity of fluorescence in the bacterial population.

2.4 Working hypothesis

As reviewed above mechanisms potentially influencing the virulence of *A. pleuropneumoniae* have been intensively researched under in vitro conditions with anaerobiosis and iron availability identified as major environmental signals triggering regulatory processes in the bacterium.

This study aims to further elucidate the mechanisms by studying them in vivo in the pig. Thus, the kinetics of i) Fur-dependent, ii) HlyX-dependent and iii) ArcA-dependent gene expression were to be elucidated in an infection experiment with a GFP marker strain or alternative methodological approaches.

3 Material and Methods

3.1 Chemicals, reagents and equipment

Chemicals and reagents used in this study were purchased from Sigma (Deisenhofen, Germany), Merck (Darmstadt, Germany) or Roth (Karlsruhe, Germany) unless stated otherwise. Media and media ingredients were purchased from Difco (Augsburg, Germany) and Oxoid (Wesel, Germany). Equipment and specific biologicals are indicated in footnotes.

3.2 Bacterial cultures

3.2.1 List of bacterial strains, plasmids and primers

Bacterial strains used in this study are listed in Table 1, plasmids are listed in Table 2, primers are listed in Table 3.

Table 1 Bacterial strains

Strain	Characteristic(s)	Source and/or reference
<i>E. coli</i> β 2155	<i>thrB1004 pro thi hsdS lacZ</i> Δ <i>M15</i> (F' <i>lacZ</i> Δ <i>M15 lacI</i> ^f <i>traD36 proA</i> ⁺ <i>proB</i> ⁺) Δ <i>dap::erm</i> (Erm ^r)	DEHIO and MEYER 1997
<i>E. coli</i> HB101	K-12 derivative, <i>supE44</i> , <i>hsd20</i> , r _B m _B , <i>recA13</i> , <i>ara-14</i> , <i>proA2</i> , <i>lacY1</i> , <i>galK2</i> , <i>rpsL20</i> , <i>xyl-5</i> , <i>mtl-1</i>	BOYER and ROULLAND-DUSSOIX 1969
<i>A. pleuropneumoniae</i> AP76	<i>A. pleuropneumoniae</i> serotype 7 isolate 76	ANDERSON et al. 1991
<i>A. pleuropneumoniae</i> Δ <i>fur</i>	Unmarked <i>fur</i> -negative knockout mutant of <i>A. pleuropneumoniae</i> AP76	JACOBSEN et al. 2005a
<i>A. pleuropneumoniae</i> Δ <i>frpB</i>	Unmarked <i>frpB</i> -negative knockout mutant of <i>A. pleuropneumoniae</i> AP76	BENDALLAH 2008
<i>A. pleuropneumoniae</i> Δ <i>ape51</i>	Unmarked <i>ape51</i> -negative knockout mutant of <i>A. pleuropneumoniae</i> AP76	This work
<i>A. pleuropneumoniae</i> Δ <i>tbpBA</i>	Unmarked <i>tbpBA</i> -negative knockout mutant of <i>A. pleuropneumoniae</i> AP76	BALTES et al. 2002
<i>A. pleuropneumoniae</i> <i>tbpB::gfp::tbpA</i>	<i>A. pleuropneumoniae</i> AP76 GFP marker strain, with insertion of <i>gfpmut3</i> gene between the genes encoding the transferrin binding proteins B (<i>tbpB</i>) and A (<i>tbpA</i>)	JACOBSEN and DRECKMANN, this work

Table 2 Plasmids

Plasmids	Characteristic(s)	Source and/or reference
pGEX5x3	<i>E. coli</i> expression vector carrying an <i>bla</i> resistance determinant, for construction of GST fusion proteins	Amersham Biosciences
pLS88	Broad-host-range shuttle vector from <i>Haemophilus ducreyi</i> ; Str ^r Sm ^r Km ^r	WILLSON et al. 1989
pEMOC2	Transconjugation vector based on pBluescript SK with <i>mobRP4</i> , a polycloning site, Cm ^r , and transcriptional fusion of the <i>omlA</i> promoter with the <i>sacB</i> gene	Accession no. AJ868288, BALTES et al. 2003b
pCR [®] 2.1-TOPO	Topoisomerase I-“enhanced” <i>E.coli</i> cloning vector carrying ampicillin and kanamycin resistance determinants as well as a <i>lacZ</i> gene for blue-white selection	TOPO TA Cloning; Invitrogen, Karlsruhe, Germany
pMW102	Vector with <i>amp</i> resistance determinant carrying the <i>gfpmut3</i> gene	BUMANN, unpublished
pTB801	Ligation of the <i>tbpB</i> PCR product with primers oTbpB1G and oTbpB2G into pCR [®] 2.1-TOPO	This work
pGFPA801	PCR products amplified with primers oGfp11/ oGfp12 and oTbpA1G / oTbpA2G were restricted with EarI, ligated, reamplified with oGfp11 and oTbpA2G and cloned into pCR [®] 2.1-TOPO	This work
pTGT801	Vectors pTB801 and pGFPA801 were restricted with BsmBI and ApaI. The <i>tbpB</i> -containing fragment from pTB801 was ligated into pGFPA801	This work
pTGT700	pEMOC2-based plasmid carrying the <i>tbpB::gfp::tbpA</i> -construct, pTGT801 and pEMOC2 were restricted with ApaI and NotI and ligated	JACOBSEN 2005, unpublished
pApe700	pEMOC2 based plasmid carrying the truncated <i>ape51</i> ORF, PCR products amplified with primers oApe-1 and oApe-2 and oApe-3 and oApe-4 respectively were digested with BsmBI and ligated; the resulting 1523 bp fragment and pEMOC2 were restricted with PspOMI and NotI and ligated with each other	This work
pApe1300a	pLS88 containing the complete <i>ape51</i> ORF in positive orientation with respect to the <i>sulA</i> promoter	This work
pApe500	Ligation of a NotI restricted PCR product generated with primers oGST-Ape-1 and oGST-Ape-2 into pGEX5x3	This work
pFrpB500	Ligation of a NotI restricted PCR product generated with primers oGST-FrpB-1 and oGST-FrpB-2 into pGEX5x3	This work
pFrpB1300	pLS88 containing the complete <i>frpB</i> ORF in positive orientation with respect to the <i>sulA</i> promoter	BENDALLAH 2008

Table 3 Primers

Primer	Characteristic(s)	Source and/or reference
oApe-1	TAGCGGGCCCCCACAATCTTGATTGTCG (upstream primer with an internal PspOMI site [underlined] comprising position 740 to 759 upstream of the <i>ape51</i> start codon)	This work
oApe-2	TAGCCGTCTCCATTACATAAAATGTCCTTTTTAGAAAGAAGATG	This work

Primer	Characteristic(s)	Source and/or reference
	(downstream primer with an internal BsmBI site [underlined] comprising position 26 upstream of the <i>ape51</i> start codon to position 2 downstream of the start codon)	
oApe-3	TAG <u>CCG</u> TCTCCTAATTATTTTTCCCTTATGACAAGCGGTCG (upstream primer with an internal BsmBI site [underlined] comprising the <i>ape51</i> stop codon TAA to position 27 downstream of the stop codon)	This work
oApe-4	TAGCGCGGCCGCACATTATCTCTCCCGCATTG (downstream primer with an internal NotI site [underlined] comprising position 730 to 745 downstream of the <i>ape51</i> stop codon)	This work
oApe-K3	TAGCGAATTCGAAGATCACCTAATACACCG (upstream primer with an internal EcoRI site [underlined] comprising position 109 to 129 upstream of the <i>ape51</i> start codon)	This work
oApe-K4	TAGCGAATTCGTATTCTACAAGCGCAAGGC (downstream primer with an internal EcoRI site [underlined] comprising position 145 to 165 downstream of the <i>ape51</i> stop codon)	This work
oApe-D2	CTTTACCTTGTAACACCG (upstream primer comprising position 200 to 219 downstream of the <i>ape51</i> start codon)	This work
oApe-RT1	CAGGTTTATTCTCTGCGTCG (upstream primer comprising position 26 to 45 downstream of the <i>ape51</i> start codon)	This work
oApe-RT2	AAACGGTAACCTAGGTTTCGC (downstream primer comprising position 2 to 21 upstream of the <i>ape51</i> stop codon)	This work
oArcA-3	GATCC <u>GTCTCT</u> TTGGAAATCGGTGCGGACG (upstream primer containing an internal BsmBI site [underlined] comprising positions 273 to 292 downstream of the <i>arcA</i> start codon)	BUETTNER 2008
oArcA-8	TACTACTCTAACTCGCCGCA (downstream primer comprising position 15 upstream the <i>arcA</i> stop codon to position 2 downstream the <i>arcA</i> stop codon)	BUETTNER 2008
oGST-Ape-1	GTCAGCGGCCGCGCCGGTCGGAAATACCTTTACC (upstream primer with an internal NotI site [underlined] comprising positions 58 to 78 downstream of the <i>ape51</i> start codon)	This work
oGST-Ape-2	GTCAGCGGCCGCGCAAGAAACGACCGCTTGTC (downstream primer with an internal NotI site [underlined] comprising positions 16 to 36 downstream of the <i>ape51</i> stop codon)	This work
oGST-FrpB-1	GTCAGCGGCCGCGTGTCCGCACAATACATTTCCG (upstream primer with an internal NotI site [underlined] comprising positions 187 to 207 downstream of the <i>frpB</i> start codon)	This work
oGST-FrpB-2	GTCAGCGGCCGCGAAGCGGTCGTAAAAACAACCG (downstream primer with an internal NotI site [underlined] comprising positions 31 to 51 downstream of the <i>ape51</i> stop codon)	This work
oGFPI1	CTAGATCGTCTCATAAGGAATTTGCAATGAGTAAAGGAGAAGAA C (upstream primer with an internal BsmBI site [underlined] comprising positions 747 to 765 of plasmid pMW 102)	JACOBSEN 2005, unpublished
oGFPI2	TTGGCGCTCTTCCTTATTATTTGTATAGTTCATCCATGCC	Jacobsen 2005,

Primer	Characteristic(s)	Source and/or reference
	(downstream primer with an internal Earl site [underlined] comprising positions 1440 to 1463 of plasmid pMW 102)	unpublished
oTbpA1G	AGTAA <u>ACTCTTC</u> CATAAGGAATTTGCAATG (upstream primer with an internal Earl site [underlined] comprising positions 1 downstream of the <i>tbpB</i> stop codon to 1 downstream of the <i>tbpA</i> start codon)	Jacobsen 2005, unpublished
oTbpA2G	ATTAG <u>CGGCCGCG</u> GAGATCCACTCTCTGAAT (downstream primer with an internal NotI site [underlined] comprising positions 684 to 701 downstream of the <i>tbpA</i> start codon)	Jacobsen 2005, unpublished
oTbpA4G	CATCGTTCAATTGCACCGCT (downstream primer comprising positions 71 to 90 downstream of the <i>tbpA</i> start codon)	Jacobsen 2005, unpublished
oTbpB1G	AATGTT <u>GGGCCCG</u> TTCGGATTATTGATGCC (upstream primer with an internal ApaI site [underlined] comprising positions 930 to 947 downstream of the <i>tbpB</i> start codon)	Jacobsen 2005, unpublished
oTbpB2G	CATTG <u>CGTCTCC</u> CTTATTATTTTTTACTTGT (downstream primer with an internal BsmBI site [underlined] comprising positions 9 to 29 upstream of the <i>tbpA</i> start codon)	Jacobsen 2005, unpublished
oTbpB3G	CGATGTAGCAGTTAGTGGTG (upstream primer comprising positions 121 to 140 upstream of the <i>tbpA</i> start codon)	Jacobsen 2005, unpublished

3.2.1.1 Growth conditions, media, antibiotic solutions and supplements

E. coli strains were cultured in Luria-Bertani (LB) medium at 37°C in an incubator¹ or a shaking incubator²; if necessary, the medium was supplemented with the appropriate antibiotics (ampicillin 100 µg/ml, chloramphenicol 25 µg/ml), and *E. coli* β2155 Δ*dapA* was supplemented with diaminopimelic acid (1 mM).

A. pleuropneumoniae strains were incubated at 37°C and 5 % CO₂ in supplemented PPLO medium with Tween[®] 80 (0.1 %). For growth and survival curves supplemented medium was inoculated with 1% of an aerobically grown log phase culture with an optical density at 600nm (OD₆₀₀) of 0.3. Aerobic cultures were grown with shaking, for anaerobic cultures the medium was preincubated for 24 h in an anaerobic chamber³ with 5 % CO₂, 10 % H₂, and 85 % N₂, the actual incubation was performed under the same conditions. For plate bioassays the strains were grown on

¹ Heraeus T6320 3PH, Heraeus Instruments GmbH Labortechnik, Hanau, Germany

² Incubator shaker Series 25, New Brunswick Scientific Co., Inc., Edison, NJ, U.S.A.

³ DonWhitley Scientific, Shipley, Great Britain

BHI agar supplemented with NAD (10µg/ml), for two-dimensional gel electrophoresis they were grown in supplemented DMEM low glucose medium¹.

For selection of *A. pleuropneumoniae* transconjugants, chloramphenicol (initially 2 µg/ml and for subculture 5 µg/ml) was added. Iron restriction was achieved by adding diethylenetriamine-pentaacetic acid calcium trisodium salt hydrate (Na₃CaDTPA). The medium for counterselection consisted of 0.4 volumes of 2.5 x PPLO broth without NaCl (Bacto Beef Heart for infusion [46 g/l], heated and filtered as recommended by the manufacturer, plus Bacto Peptone [9.25 g /l]), 0.5 volume of sucrose (40%) and 0.1 volume of equine serum. The cultures were incubated at 37°C in a 5% CO₂ incubator² or in a shaking incubator.

Media

LB broth	10 g Bacto [®] tryptone, 5 g yeast extract, 5 g NaCl, add distilled water to 1 litre, autoclave
LB agar	LB broth with 1.5 % agar (w/v), autoclave
PPLO broth	21 g PPLO [®] broth, add distilled water to 1 litre, sterile filter; prior to use EIVX (see below) and Tween [®] 80 (0.1% final concentration) were added
PPLO agar	35 g PPLO [®] agar, 3 g agar, add distilled water to 1 litre, autoclave, prior to use EIVX (see below) was added
Supplemented blood agar	40g Columbia blood agar base [®] , add distilled water to 800 ml, autoclave, cool to 55°C, add 200 ml bovine blood and 0.7% nicotine amide adenine dinucleotide (NAD)

¹ Invitrogen, Karlsruhe, Germany

² Heraeus CO₂-Auto-Zero, Heraeus Instruments, Hanau, Germany

Selective blood agar Add crystal violet (1 µg/ml), lincomycin (1 µg/ml), nystatin (50 µg/ml), bacitracin (100 µg/ml) to supplemented blood agar

Antibiotic solutions and supplements

Ampicillin stock solution 100 mg/ml in 70 % ethanol, a few drops of concentrated HCl were added until all substance was completely dissolved

Bacitracin stock solution (100 mg/ml) in A. bidest.

Chloramphenicol stock solution (100 mg/ml) in 70% ethanol

Lincomycin stock solution (1 mg/ml) in A. bidest.

Kanamycin stock solution (50 mg/ml) 50% glycerol

Nystatin stock solution (50 mg/ml) in A. bidest.

Crystal violet stock solution (1 mg/ml) in A. bidest.

Diaminopimelic acid stock solution (100 mM) in A. bidest., a few drops of concentrated HCl were added until the solution cleared

PPLO supplement stock solution (EIVX), 100 x concentrated

1 g/l L-glutamine¹, 26 g/l L-cysteine dihydrochloride, 1 g/l L-cystine dihydrochloride, 1 g/l NAD in 10% D (+) glucose monohydrate

¹ Serva, Heidelberg, Germany

All stock solutions were sterilised by filtration¹ and stored at -20°C.

3.3 Bacteriological methods

3.3.1 Plate bioassay: Application of iron from different sources under iron-restricted conditions

To induce iron restriction Na_3CaDTPA was spread onto NAD (10 $\mu\text{g/ml}$)-supplemented BHI agar plates to a final concentration of 300 μM . Bacteria were grown in supplemented PPLO medium in a shaking incubator to an optical density at OD_{600} of 0.3-0.4, iron restriction was induced with 150 μM Na_3CaDTPA , and the cultures were further incubated for 1 h. Bacteria were diluted 1:100 with 0.85 % NaCl, and 200 μl were evenly spread onto the iron-restricted plates. Paper discs (diameter 5 mm) soaked with 15 μl ferric citrate (Ferric nitrate 4 mM, sodium citrate 2 mM) served as positive control, and discs soaked with 15 μl HEPES-sodiumbicarbonate buffer as a negative control. To investigate the use of porcine transferrin as iron source, discs soaked with 23 μl transferrin (1.9 mM) were placed onto the plates and incubated overnight at 37°C in a 5 % CO_2 incubator.

3.3.2 Determination of bacterial growth by determination of optical density and colony forming units

Two ml of overnight culture were inoculated in 18 ml PPLO broth, grown to an OD_{600} of approximately 0.3 in the shaking incubator at 37°C and 200 rpm; 2 ml of that culture were inoculated in 200 ml PPLO broth and the OD_{600} was determined every 30 to 120 min until stationary phase was reached. The number of colony forming units (cfu) was determined via serial dilution and subsequent plating on supplemented PPLO agar plates.

¹ FP30/o,2 CA-S, pore size 0.2 μm , cellulose acetate, Schleicher & Schuell, Dassel, Germany

3.3.3 Urease assay

To test whether a bacterial colony is urease positive, bacterial colonies were overlaid with 10 ml of urease test agarose (agarose [0.5 %], urea [20 mg/ml], phenol red [100 µg/ml]) per agar plate. A magenta colony color and halo indicating urease activity was visible after 1-5 min, negative colonies turned yellow.

3.4 Manipulation of nucleic acids

3.4.1 Plasmids

The plasmids used in this study are summarised in Table 2. Standard protocols and the respective manufacturer's instructions were applied to restriction endonuclease digests, ligations and agarose¹ gel electrophoresis. Restriction endonucleases and other enzymes were purchased from New England Biolabs, Frankfurt, Germany, DNA size standards and buffers were purchased from Diagonal, Münster, Germany unless stated otherwise. Plasmids were analysed by gel electrophoresis in 0.5 × TBE buffer (see below) on 0.8 % or 1.5 % agarose gels; for preparative gels 1 × TAE buffer (see below) was used.

10 × TBE buffer 1 M Tris-borate, 10 mM EDTA (pH 8.0)

50 × TAE buffer 2 M Tris-HCl, 1 M acetic acid, 50 mM EDTA (pH 8.0)

3.4.2 Primers

The primers used in this study are summarised in Table 3. Primers were synthesised by Invitrogen, Karlsruhe, Germany and Biomers, Ulm, Germany.

¹ Biozym, Hess. Oldendorf, Germany

3.5 Isolation of nucleic acids

3.5.1 Plasmid DNA

Preparation of plasmid DNA was either done by alkaline lysis (BIRNBOIM and DOLY 1979) following standard procedures (SAMBROOK et al. 1989) or by using the NucleoBond[®] AX kit¹ according to the manufacturer's instructions.

DNA cleanup following alkaline lysis was performed by using the Nucleo Spin Extract II² kit according to the manufacturer's instructions. Centrifugation steps were carried out in a microcentrifuge³.

3.5.2 Total chromosomal DNA of *A. pleuropneumoniae*

An overnight culture (250 ml) grown in a shaking incubator was harvested by centrifugation⁴ at 10,400 x g for 10 min. To lyse the cells the pellet was resuspended in 1.5 ml solution 1 (150 mM NaCl, 100 mM EDTA, 3 mg/ml lysozyme), incubated at 37°C for 15 min, and then frozen at -70 °C overnight. Afterwards 12.5 ml of solution 2 (100 mM NaCl, 100 mM Tris/HCl pH 8.0, 1 % sodium dodecyl sulphate [SDS]) were added and the tube was thawed at 55 °C, followed by an additional freezing and thawing cycle to obtain complete bacterial lysis. The solution was kept on ice, 3 ml of Roti[®]-phenol were added, mixed by repeated and careful inversion of the tube and again frozen at -70 °C for 0.5 h. After thawing at 55 °C the lysate was centrifuged at 16,925 x g for 15 min at 4 °C again. The supernatant was transferred to a fresh tube; 2.5 volumes of ice-cold ethanol were added and mixed by repeated careful inversion of the tube. Precipitating DNA was transferred to a new tube and resuspended in 5 ml TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA [pH 8.0]). RNase was added to a final concentration of 10 mg/ml and incubated at 37°C for 10 min; 1 ml Roti[®]-phenol was added, the phases were mixed by gentle inversion and frozen at -70°C for 30 min. The preparation was thawed, 0.5 ml of chloroform-isoamyl alcohol (24 : 1) were

¹ Macherey & Nagel, Düren, Germany

² Macherey & Nagel, Düren, Germany

³ Eppendorf, Centrifuge 5415 D, Hamburg, Germany

⁴ Sorvall RC-5B Refrigerated Superspeed Centrifuge, Du Pont Inst., Bad Homburg, Germany

added, and the mixture was centrifuged at 13,000 x g for 10 min at 4°C. Chloroform-isoamyl extraction was repeated at least 3 times until the interphase became clear. The upper phase was transferred into a new tube, and DNA was precipitated by adding Na-acetate (pH 5.2) to a final concentration of 300 mM and 2.5 volumes of ethanol. The DNA thread generated by careful inversion of the tube was collected with a small pipette tip and washed in 70 % ethanol for 5 min. DNA was dissolved in 500 µl A. bidest., and 5 µl of DNA were analysed by agarose gel electrophoresis.

3.5.3 Isolation of RNA

Liquid cultures were grown to an OD₆₀₀ of 0.4 to 0.6, and 25 ml were harvested by centrifugation¹ at 7,200 x g for 10 min at 4 °C. The pellet was frozen at -70 °C for 0.5 h and subsequently processed with the FAST RNA Pro Blue kit² according to the manufacturer's recommendations. Further RNA cleanup was performed using the protocol provided with the RNeasy[®] Mini Kit³ according to the manufacturer's instructions. Quality of RNA was confirmed on a glyoxal / dimethyl sulfoxide gel according to standard procedures (SAMBROOK et al. 1989).

3.5.4 Polymerase chain reaction

Polymerase chain reactions (PCRs) were performed in a thermal cycler⁴ in 25 or 50 µl total reaction volumes using *Taq* DNA polymerase⁵. The reaction mixtures were prepared on ice by addition of the reagents in the order described in Table 4. In case of transformants, single colonies were lysed in 100 µl A. dest., and 5 µl were then used as DNA template. PCR conditions are listed in

Table 5; the extension time was estimated based on the length of the expected PCR product (1 min for every 1 kb). PCR products were analysed by gel electrophoresis in 0.5 x TBE buffer on a 1.5 % agarose gel if products were > 1500 base pairs (bp), otherwise a 0.8 % agarose gel was used.

¹ Eppendorf , Centrifuge 5810R, Hamburg, Germany

² MP Biomedicals, Illkirch, France

³ Qiagen, Hilden, Germany

⁴ Eppendorf Mastercycler, Eppendorf AG, Hamburg, Germany

⁵ Invitrogen, Eggenstein, Germany

Table 4 Components for PCR.

Reaction components	Stock solution	Final concentration	Volume per reaction (µl)	Volume per reaction (µl)
A. bidest.	-	-	11.15	22.3
MgCl ₂	50 mM	1.5 mM	0.75	1.5
PCR-buffer	10 x	1 x	2.5	5
dNTPs	10 mM	0.2 mM	0.5	1
forward primer	5 pmol/µl	0.5 pmol/µl	2.5	5
reverse primer	5 pmol/µl	5 pmol/µl	2.5	5
<i>Taq</i> DNA polymerase	5 U/µl	0.5	0.1	0.2
template	-	-	5	10
final volume			25	50

Table 5 PCR conditions used in this study.

Primers	PCR protocol
oApe-1, oApe-2, oApe-3, oApe-4	3' 94°C, (30" 94°C, 1' 52°C, 2' 30" 72°C) x 32, 1 0' 72°C
oApe-K3, oApe-K4	3' 94°C, (30" 94°C, 1' 56°C, 1' 7 2°C) x 32, 10' 72°C
oApe-K3, oApe-D2	3' 94°C, (30" 94°C, 1' 53°C, 30" 72°C) x 32, 10' 72°C
oGST-Ape-1, oGST-Ape-2	3' 94°C, (30" 94°C, 1' 56°C, 40" 72°C) x 32, 10' 72°C
oGST-FrpB-1, oGST-FrpB-2	3' 94°C, (30" 94°C, 1' 57°C, 2' 72°C) x 32, 10' 72 °C
oApe-RT1, oApe-RT2, oArcA-3, oArcA-8	3' 94°C, (30" 94°C, 1' 55°C, 30" 72°C) x 32, 10' 72°C
oTbpA1G, oTbpA2G, oGfpI1, oGfpI2, oTbpB1G, oTbpB2G	3' 94°C, (30" 94°C, 1' 57°C, 30" 72°C) x 32, 10' 72°C
oGfpI1, oTbpA2G	3' 94°C, (30" 94°C, 1' 45" 57°C, 30" 72°C) x 32, 10' 72°C
oTbpB1G, oTbpA2G, oTbpB3G, oTbpA4G	3' 94°C, (30" 94°C, 1' 55°C, 2' 30" 72°C) x 32, 1 0' 72°C

3.5.5 Preparation of PCR template from bacterial colonies

A single colony was touched with a small sterile glass bead which was then transferred into 100 µl A. dest. in a microtiter plate or in a 1.5 ml reaction tube. The solution was either boiled in a microwave for 8 min at 180 W or stored at room temperature for 15 min. As a negative control 5 µl A. dest. served as template in a 25 µl PCR reaction.

3.5.6 Reverse transcriptase polymerase chain reaction

Total *A. pleuropneumoniae* wt RNA was prepared, and residual DNA was removed by applying the Turbo DNA-free™¹ protocol for rigorous DNase treatment to approximately 10 µg RNA. Reverse Transcription was performed following the manufacturer's protocol in the manual for the Superscript® II Reverse Transcriptase Kit² including a final RNase H treatment to remove RNA complementary to the cDNA. Approximately 5 µg of DNase-treated RNA and 5 pmol of gene-specific reverse primer (Table 3) were used in the 1st strand synthesis. For each sample a negative control was included to which no Superscript® II Reverse Transcriptase was added in order to verify efficient DNase treatment. The obtained cDNAs were purified using the NucleoSpin® Extract II kit and eluates served as templates in the subsequent PCR reactions. Primer pairs were positioned within the *arcA* gene (oArcA-3 and oArcA-8) for amplification of an *arcA* specific RNA, upstream of the *arcA* stop codon and downstream of the *ape51* start codon to amplify the passage between the two genes (oApe-K3 and oApe-D2) and within the *ape51* gene itself (oApe-RT1 and oApe-RT2). For primer details see Table 3.

3.5.7 Pulsed field gel electrophoresis (PFGE)

3.5.7.1 Isolation of agarose-embedded chromosomal *A. pleuropneumoniae* DNA

A. pleuropneumoniae was grown on supplemented PPLO agar and washed off the plate with 3 ml of PETT IV-buffer (see below). Five ml of the bacterial suspension

¹ Ambion/Applied Biosystems, Darmstadt, Germany

² Invitrogen, Karlsruhe, Germany

were adjusted to an OD₆₀₀ of 0.3, and then centrifuged at 3,800 x g¹ at 4 °C for 10 min, the supernatant was removed and the cells were washed in 5 ml ice-cold PETT IV-buffer. After centrifugation the pellet was suspended in 0.5 ml PETT IV buffer and incubated briefly at 55 °C before 0.5 ml of 1.2 % agarose² (kept at 55 °C) were added and mixed thoroughly. The suspension was poured into 100 µl plug molds (Sample CHEF Disposable Plug Mold³) and allowed to solidify at 4 °C for 10 to 15 min. The agarose plugs were removed from the mold and 5 plugs each were transferred into a polypropylene tube containing 3 ml lysis buffer (see below). The tube was incubated horizontally at 37 °C for 2 h after which the lysis buffer was discarded. Three ml EPS buffer (see below) were added, and the plugs were incubated at 55 °C overnight. The EPS buffer was discarded and the plugs were washed twice with 3 ml A. bidest. for 15 min by tube-rolling at room temperature, then the A. bidest. was discarded. To inactivate residual proteinase K, plugs were washed twice with 2 ml TE-PMSF (see below) for 30 min at room temperature. Plugs were then washed with 3 ml A. bidest. for 15 min, which was removed before washing the plugs with 3 ml TE buffer for 30 min and finally storing them in 5 ml TE buffer at 4 °C.

PETT IV-buffer	1 M NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA
lysis buffer	1 M NaCl, 10 mM Tris-HCl (pH 8.0), 0.2 M EDTA, 0.5 % <i>N</i> -laurylsarcosine, 0.2 % sodium deoxycholate, 2 µg/ml RNase, 1 mg/ml lysozyme
EPS	0.5 M EDTA, 1 % <i>N</i> -laurylsarcosine, 1 mg/ml proteinase K
TE-PMSF	1.5 mM PMSF in TE buffer
PMSF	17 mg phenylmethylsulfonyl fluoride ⁴ per ml isopropanol

3.5.7.2 Restriction endonuclease digestion of DNA embedded in agarose plugs

One half of a gel plug was used for each reaction. Prior to digestion, plugs were equilibrated in three volumes of an appropriate restriction endonuclease buffer (supplied by the manufacturer) for 1 h at room temperature. The buffer was removed,

¹ Eppendorf, Centrifuge 5810R, Hamburg, Germany

² Biozym Scientific, Hess. Oldendorf, Germany

³ BioRad, Munich, Germany

⁴ Serva, Heidelberg, Germany

new buffer was added, and 10 U of enzyme were used to digest the DNA in the plugs overnight at the temperature appropriate for the respective restriction endonuclease¹.

3.5.7.3 Pulsed field gel electrophoresis

A 0.8 % agarose² gel was prepared using 0.5 x TBE buffer, cooled to 55 °C and poured into a gel casting platform³. The gel was allowed to solidify for 15 min before removal of the comb. The gel plugs containing the digested DNA were transferred into the slots. Bacteriophage lambda concatamers embedded in agarose gel were used as size marker⁴. The slots were filled up with agarose gel to prevent upwards migration of the plugs. The gel was placed into the electrophoresis chamber⁵ and carefully immersed in cold (12 °C) 0.5 x TBE buffer avoiding displacement of the gel from the gel tray. Gels were run at 6 V/cm and 12 °C with linear ramped switch times from 10 to 20 sec for 14 h and from 35 to 70 sec for 12 h. After the run, DNA was stained with ethidium bromide in A. bidest. (0.5 µg/ml) for 15 min and destained in A. bidest. for 15 to 30 min. Gels were documented on a UV transilluminator and photographed with an image documentation system⁶.

3.5.8 Nucleic acid detection

3.5.8.1 Southern blotting

DNA was digested with an appropriate restriction endonuclease and separated by gel electrophoresis. The gel was photographed with a fluorescent ruler on a UV transilluminator prior to Southern transfer by capillary blotting onto nitrocellulose membrane⁷ according to standard procedures (SAMBROOK et al. 1989). DNA

¹ New England Biolabs, Ipswich, USA
² Biozym Scientific, Hess. Oldendorf, Germany
³ BioRad, Munich, Germany
⁴ New England Biolabs, Ipswich, USA
⁵ CHEF-DR III pulsed-field electrophoresis system, Bio-Rad, Munich, Germany
⁶ BioRad, Munich, Germany
⁷ Positive membrane, MP Biomedicals, Illkirch, France

crosslinking was achieved by baking the membrane in an oven¹ at 80°C for 120 minutes.

3.5.8.2 Labelling of DNA probes with $\alpha^{32}\text{P}$ -dCTP

Random priming of PCR products was used to generate DNA probes. Briefly, 20 to 30 ng of DNA in 5 μl A. bidest. were denatured at 100°C for 5 min and placed on ice. Then, 10.5 μl of A. bidest., 5 μl OLB solution, 1 μl acetylated BSA (10 mg/ml), 1 μl Klenow fragment (5 U/ μl) and 2.5 μl $\alpha^{32}\text{P}$ -dCTP (370 kBq/ μl) were added and the reaction was incubated at room temperature for 4 h. One hundred μl of stop solution were added to stop the reaction. The labelled DNA probe was used right away. Directly before using the labelled DNA probe it was denatured at 95°C for 5 min.

OLB solution	100 μl solution A, 250 μl solution B, 150 μl solution C
Solution O	625 μl 2 M Tris-HCl (pH 8), 125 μl 1M MgCl, 250 μl A. bidest.
Solution A	1 ml solution O, 18 μl mercaptoethanol, 5 μl 100 mM dTTP, 5 μl 100 mM dGTP, 5 μl 100 mM dATP
Solution B	2 M HEPES (pH 6.6)
Solution C	random hexa-desoxynucleotides in TE buffer, 4.5 mg/ml ²
Stop solution	20 mM NaCl, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA (pH 8), 0.25% [w/v] SDS

3.5.8.3 Southern hybridisation

Southern hybridisation was performed overnight following standard procedures (SAMBROOK et al. 1989) in a hybridisation oven³ at 60 °C, using a hybridisation buffer containing 6 x SSC (see below), 0.5 % SDS, 5 x Denhardt's solution (see below), and 0.01 M EDTA. Blots were washed three times for 30 minutes at 65 °C (3 x SSC, 0.5 % SDS), and exposed to an X-ray film⁴ which was stored at -70 °C.

¹ Booskamp, Wuppertal, Germany

² Amersham Biosciences, Piscataway, USA

³ Mini hybridization oven, Qbiogene, Heidelberg, Germany

⁴ Kodak X-OMAT[®] AR or BioMax, SIGMA, Deisenhofen, Germany

Exposure time was adjusted depending on the signal strength, in this case, overnight.

20 × SSC	3 M NaCl, 0.3 M trisodium acetate
50 × Denhardt's solution	1 % [w/v] polyvinylpyrrolidone, 1 % [w/v] Ficoll 400, 1 % [w/v] BSA; in A. bidest.

3.5.9 Nucleotide sequencing and sequence analysis

Nucleotide sequencing was done by SEQLAB, Göttingen, Germany. Sequence analyses were performed using **Basic Local Alignment Search Tool (BLAST)**.

3.5.10 Transformation

3.5.10.1 Preparation of electro-competent *E. coli* cells for transformation

Competent *E. coli* cells were prepared using the method described by TUNG and CHOW (1995). Two Erlenmeyer flasks each with 250 ml LB broth were inoculated with 2.5 ml each of an overnight culture of *E. coli* cells, grown to an OD₆₀₀ of 0.6 with vigorous agitation and chilled on ice for 30 min. Cells were harvested by centrifugation at 2,600 x g at 4 °C for 15 min. The obtained pellets were resuspended in 5 ml pre-chilled washing buffer (10 % [v/v] glycerol in A. bidest.), and 100 ml of additional washing buffer was added. Cells were again pelleted by centrifugation at 9,200 x g and 4 °C for 15 min, and the washing step was repeated twice. After the last washing step, both pellets were suspended in a total volume of 5 ml pre-chilled GYTT medium (see below). Cells were transferred to pre-chilled 1.5 ml reaction tubes and stored in 200 µl aliquots at -70 °C until use.

GYTT medium	10 % [v/v] glycerol, 0.125 % [w/v] yeast extract, 0.25 % [w/v] Bacto [®] tryptone, 0.02 % [v/v] Tween [®] 80
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3.5.10.2 Electrotransformation of *E. coli*

For transformation of electro-competent *E. coli* cells were thawed on ice, and 30 to 50 µl were mixed with 10 µl of a dialysed ligation reaction. The Gene Pulser¹ was set to 2.5 kV and 25 µF, the pulse controller¹ was set on 200 Ohm. Transformation of the DNA-cell-mix was performed according to the manufacturer's instructions in a 0.2 cm electroporation cuvette². After transformation 500 µl prewarmed LB medium was added, mixed well and transferred to a 1.5 ml reaction tube. In order to express the respective antibiotic resistance determinant, the cells were incubated at 37 °C for 1 h with shaking and then plated on LB plates with the supplemented antibiotic.

3.5.10.3 Preparation of electro-competent *A. pleuropneumoniae*

A modified method by FREY (1992) and TUNG and CHOW (1995) was used for preparation of electro-competent *A. pleuropneumoniae*. 250 ml of supplemented PPLO broth with Tween[®] 80 (0.1 %) was inoculated with 25 ml of an overnight culture and incubated with shaking to an OD₆₀₀ of 0.3 to 0.4. The culture was chilled on ice for 20 to 30 min. The bacteria were harvested by centrifugation at 5000 x g, 4 °C for 10 min. The pellet was washed three times in 50 ml, 30 ml and 20 ml GYTT medium respectively. Centrifugation was accomplished at 5000 x g, 4 °C for 10 min. After the last washing step competent bacteria were resuspended in 2 ml GYTT medium. Electrotransformation was performed on the same day.

3.5.10.4 Electrotransformation of *A. pleuropneumoniae*

An aliquot of 250 µl of competent bacteria was mixed with 2 to 5 µl of salt free DNA in a pre-cooled tube. The mixture was kept on ice for 30 min., then it was transferred to a cold electrotransformation cuvette³. The gene pulser⁴ was set to 2.5 kV, 25 µFD and 800 Ohm and the pulse was applied. One ml of prewarmed (37 °C), supplemented PPLO broth was added immediately afterwards. The mixture was

¹ Bio-Rad, Munich, Germany

² Electrotransformation cuvette, 0.2 cm electrode gap, Cellprojects, Harrietsham, UK

³ Electrotransformation cuvette, 0.2 cm electrode gap, Cellprojects, Harrietsham, UK

⁴ Bio-Rad, Munich, Germany

transferred to a glass tube and the bacteria were allowed to regenerate for 2 to 4 hours at 37 °C and 5 % CO² before plating them on supplemented PPLO agar plates with the appropriate antibiotic.

3.5.11 Construction of *A. pleuropneumoniae* isogenic deletion mutants

Deletion mutants were constructed using the method of OSWALD et al. (1999). In principle deletions were introduced into *A. pleuropneumoniae* via allelic exchange. Homologous recombination facilitated the replacement of the complete gene with a truncated version. A recombinant plasmid, harbouring the truncated version, was transferred by conjugation from an auxotrophic *E. coli* donor to the *A. pleuropneumoniae* recipient.

3.5.11.1 Conjugation of *E. coli* (donor) and *A. pleuropneumoniae* (recipient) by filter mating technique

For the construction of a deletion mutant the truncated gene was cloned into mutagenesis vector pEMOC2 to be mobilised from *E. coli* β2155, the diaminopimelic acid auxotrophic donor. *A. pleuropneumoniae* AP76 served as recipient. After donor and recipient were grown on the appropriate solid media overnight the colonies were collected with a sterile cotton swab and resuspended in TMN buffer (see below). OD₆₀₀ was adjusted to 2. It is essential not to vortex the suspension containing *E. coli* β2155 because this might damage the pili necessary for conjugation in the donor strain.

Three nitrocellulose discs¹ (0.45 µM pore size, 2.5 cm diameter) were put on sterile filter paper in a petri dish. A solution of 50 µl donor and 350 µl recipient was mixed gently by repeated pipetting and then transferred on the nitrocellulose discs. The petri dish was kept at 37 °C for the buffer to be absorbed; afterwards the discs were transferred onto prewarmed supplemented PPLO agar plates with diaminopimelic acid (1 mM), and MgSO₄ (10 mM), and incubated at 37 °C and 5 % CO₂ for 7 h. Following incubation the discs were placed into Eppendorf tubes and washed by

¹ Millipore, Eschborn, Germany

prepared lysozyme solution (10 mg/ml in 250 mM Tris [pH 8.0]) was added and the solution was kept on ice for 15 min. Two ml *N*-laurylsarcosine solution (5 %) was added to obtain a final concentration of 2 %. The cells were treated with ultrasound¹ at setting 3 and 40 % duty cycle for 2 x 3 min. Cell debris was removed by centrifugation for 15 min at 18,200 x g at 4 °C. In order to separate the periplasm and the cytoplasm from the outer membranes, the supernatant was ultracentrifuged for 1 hour at 177,000 x g² and 4 °C. The pellet containing the outer membranes was resuspended in 200 µl A. dest.

3.6.1.2 Preparation of proteins from culture supernatant

A. pleuropneumoniae overnight cultures were grown in supplemented PPLO broth at 37 °C with 5 % CO₂; 500 ml DMEM low glucose medium supplemented with PPLO-supplement was inoculated with 5 ml of an overnight culture. The cultures were either incubated under aerobic conditions at 37 °C with shaking³ at 200 rpm or under anaerobic conditions⁴ for 24 hours. In order to harvest the proteins from the supernatant the cultures were centrifuged at 9,100 x g for 15 min. The pellet was discarded and the remaining liquid was concentrated by ultrafiltration in a membrane-based system⁵ with a cut-off for proteins smaller than 10 kDa. The culture was concentrated to 50 ml, and trichloroacetic acid (TCA) was added to a final concentration of 20 %. The samples were kept at 4 °C overnight to facilitate protein precipitation. The solution was centrifuged at 9,100 x g for 15 min. The precipitated proteins were washed twice in 100 % acetone and then dissolved in 2 ml standard cell lysis buffer (SCLB; 30 mM Tris, 2 M thiourea, 7 M urea, 4 % [w/v] CHAPS, pH 8.5). Further washing with an additional 2 ml SCLB was conducted in membrane-based centrifugal devices⁶ according to the manufacturers recommendations. The remaining concentrate was used for two dimensional polyacrylamide gel electrophoresis (2 D PAGE).

¹ Sonic Cell Disruptor, Branson Sonifer, Branson Power, Dannbury, USA

² Ultracentrifuge, Beckman Coulter™, Krefeld, Germany

³ Incubator shaker Series 25, New Brunswick Scientific, Edison, USA

⁴ Anaerobic chamber, Linde, Höllriegelskreuth, Germany

⁵ Diaflo ultrafiltration membranes, Amicon, Billerica, USA

⁶ Amicon, centricon, Billerica, USA

3.6.1.3 Preparation of glutathione-S-transferase (GST) fusion proteins

1.5 ml LB medium with ampicillin (100µg/ml) was inoculated with one colony of the strain expressing the respective GST fusion protein. The culture was incubated at 37 °C with shaking until it became slightly turbid. Then it was divided, and one half was induced with 15 µl isopropyl β-D-1-thiogalactopyranoside (IPTG) (100 mM) and further incubated for another 2 h. The culture was then centrifuged at 13,000 x g for 5 min. and the pellet was resuspended in 20 to 100 µl A. dest. depending on the yield.

3.6.1.4 Preparation of GST fusion protein aggregates

In this study GST fusion protein aggregates were used to raise antibodies against *A. pleuropneumoniae* proteins in rabbits. The expression plasmids were constructed by inserting the respective genes without their signal sequence into expression vector pGEX5x3 (Table 2). In order to produce GST fusion proteins the plasmid's *tac* promoter was induced with IPTG. The resulting fusion proteins formed as inclusion bodies (aggregates) in the cytoplasm of *E. coli* were used for immunisation of rabbits after purification and solubilisation.

For preparation of GST protein aggregates 50 ml of LB broth supplemented with ampicillin (100µg/ml) were inoculated with 0.5 ml overnight culture of the *E. coli* HB101 carrying the respective expression plasmid (Table 2.) The culture was grown to an OD₆₀₀ of 0.3 to 0.5, then induced with IPTG (1 mM) and further incubated for 2 h with shaking. Bacterial cells were harvested by centrifugation at 6,000 x g for 10 min. The pellet was resuspended in 2.5 ml 25 % sucrose in 50 mM Tris (pH 8.0). The solution was frozen at -70 °C for 30 min and thawed, then 0.25 ml lysozyme (10 mg/ml in 250 mM Tris [pH 8.0]) were added and the mixture was kept on ice for 10 min. Another incubation step on ice for 10 min. followed after addition of 10 ml 2 x RIPA/TET (see below; mixed in a ratio of 5:4). The solution was sonicated¹ for 3 min using the maxi tip with output setting 5 and 50 % duty cycle; sonication was repeated until the solution appeared clear and opalescent. Sonication was followed by centrifugation at 32,500 x g for 20 min. The resulting pellet was resuspended in 500

¹ Sonic Cell Disruptor, Branson Sonifer, Branson Power, Dannbury, USA

μ l A. dest. The pellet was diluted 1:2 in 2 x sample buffer and 500 μ l of each protein preparation were separated on a 10.8 % SDS gel. The respective protein band was excised from the gel and the protein was purified via electroelution.

2X RIPA 20 mM Tris, pH 7.4
 300 mM NaCl
 2 % Na-deoxycholic acid sodium salt
 2 % NP-40[®] (Tergitol)

TET 100 mM Tris, pH 8.0
 50 mM EDTA, pH 8.0
 2 % Triton-X 100

Sample buffer (2x) 1.5 ml 0.5 M Tris pH 6.8
 6.0 ml 10 % SDS
 3.0 ml 50 % glycerole
 10.0 μ l 1 % bromophenol blue
 1.0 ml 2-mercaptoethanol

3.6.1.5 Electroelution of proteins

For elution of proteins from SDS gels the respective band was excised from the gel, placed in a cellulose based dialysis tube with a cut off of 4 to 6 kDa and emerged in SDS-buffer. Electroelution was conducted in an ice chilled electrophoresis chamber at 8.7 V/cm for 1.5 h. The eluate was concentrated in centrifugal devices according to the manufacturer's recommendations (see 3.5.1.2). After determination of protein concentration the eluate was used for immunisation of New Zealand White rabbits.

SDS buffer Tris 3.0 g
 Glycine 14.4 g
 SDS 1.0 g
 A. dest. ad 1000 ml

3.6.2 Determination of protein concentration

Protein concentrations were determined using the Micro BCA[®] Protein Assay¹ in 96 well microtiter plates according to the manufacturer's instructions. Protein samples were diluted appropriately. The assay was read in the TECAN GENios Pro reader² and quantified using the Magellan[™] software.

3.6.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Discontinuous SDS-PAGE was performed following standard procedures (SAMBROOK et al. 1989) using a Protean II Minigel system³.

3.6.4 Two-dimensional-polyacrylamide gel electrophoresis (2D-PAGE)

Two-dimensional-polyacrylamide gel electrophoresis was performed with proteins from culture supernatant prepared as described in 3.6.1.2. For first dimension focussing protein preparations were mixed with an equal volume of sample rehydration buffer (7 M urea, 2 M thiourea, and 4 % [wt/vol] CHAPS, 2 % (vol/vol) IPG buffer⁴, 2 % [wt/vol] dithiothreitol). Insoluble material was removed by centrifugation at 16,000 x g for 5 min in a microcentrifuge. Immobiline DryStrips pH 3-11 (24 cm)⁴ were rehydrated with 450 µl of rehydration buffer per strip containing 2 M thiourea, 7 M urea, 4 % (wt/vol) CHAPS, 1 % (vol/vol) IPG buffer⁴, and 0.2 % (wt/vol) dithiothreitol for 12-18 h using the Immobiline DryStrip Reswelling Tray⁴. Strips were transferred into the Ettan[™] IPGphor[™] Cup Loading Manifold⁴, and samples were loaded into anodal sample cups and subsequently focused using an Ettan[™] IPGphor[™]⁴ for 21 h in the following series of time blocks with increasing voltage: 3 h at 150 V, 3 h at 300 V, 6 h at a 1000 V gradient, 3 h at an 8000 V gradient, and 6 h at 8000 V. For second dimension, the strips were equilibrated twice for 15 min with shaking in a solution of 100 mM Tris-HCl (pH 8.0), 6 M urea, 30 % (vol/vol) glycerol,

¹ Pierce, Rockford, USA
² Tecan Group, Männedorf, Switzerland
³ BioRad, Munich, Germany
⁴ Amersham Biosciences, Piscataway, USA

and 2 % (wt/vol) SDS supplemented with 0.5 % (wt/vol) DTT for the first equilibration step and 4.5 % (wt/vol) iodoacetamide for the second equilibration step.

For the second dimension the Immobiline DryStrips were placed on top of 12.5 % SDS-PAGE containing 12.5 % (vol/vol) acrylamide and 0.33 % bisacrylamide¹ that had been precasted using an EttanTM DALTsix gel caster². The second-dimension SDS-PAGE was carried out at 12 °C and 50 V for 3 h followed by 100 V for 15 h using the EttanTM DALTsix electrophoresis system.²

3.6.5 Protein detection

3.6.5.1 Protein stains

2D gels and SDS-PAGE gels were stained with colloidal Coomassie Brilliant Blue R250 over night, destaining was done in A. dest.

3.6.5.2 Western blotting

Western blotting of the SDS-PAGE minigels was done using the Mini Trans-Blot[®] system³. Proteins were transferred to a nitrocellulose membrane⁴ as described by SAMBROOK et al. (1989) for 30 min at 50 V.

3.6.5.3 Immunoblotting using alkaline phosphatase-conjugated antibodies

Immunoblotting was performed according to standard procedures using an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody⁵, diluted 1:5,000, as conjugate, and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) as substrate for visualisation of protein bands (SAMBROOK et al. 1989). Rabbit and pig derived sera were used as indicated in Table 6 .

¹ Serva, Heidelberg, Germany
² Amersham Biosciences, Piscataway, USA
² see above
³ BioRad, Munich, Germany
⁴ Protran BA85 0.45 µM, Schleicher and Schuell, Dassel, Germany
⁵ Dianova, Hamburg, Germany

Table 6 Sera used in this study.

antiserum	characteristic	dilution	reference
anti-GST-FrpB	antibodies raised against recombinant GST-FrpB fusion protein from <i>A. pleuropneumoniae</i> serotype 7	1:1000	This work
anti-GST-Ape51	antibodies raised against recombinant GST-Ape51 fusion protein from <i>A. pleuropneumoniae</i> serotype 7	1:1000	This work
anti-outer membrane	antibodies raised against an outer membrane preparation of <i>A. pleuropneumoniae</i> AP76	1:1000	This work
anti reconvalescent	serum of pigs reconvalescent from <i>A. pleuropneumoniae</i> AP76 infection collected on day 21 p.i.	1:1000	This work

3.6.5.4 Whole cell immuno dot blot

1 ml of overnight culture of the respective strain was centrifuged at 7,500 rpm in a microcentrifuge and the pellet was resuspended in 10 % formaldehyde. The mixture was kept at room temperature over night to facilitate proper inactivation. Subsequently the cells were washed in A. dest. twice. The inactivated bacteria were dropped on nitrocellulose membranes. Further immunoblotting was conducted according to standard procedures (see 3.6.5.3).

3.6.6 Preparation of antisera

Antisera against recombinant *A. pleuropneumoniae* Ape51, FrpB proteins and *A. pleuropneumoniae* outer membranes were raised in New Zealand White rabbits. Porcine sera of reconvalescent pigs infected with *A. pleuropneumoniae* AP76 were collected on day 21 p. i. Rabbits were immunised intradermally with 100 µg protein (see above) plus 100 µl PBS mixed with 150 µl Emulsigen^{®1} per immunisation.

¹ MVP Laboratories, Omaha, USA

Timeline for immunisation

Day 0	First immunisation with antigen preparation, sampling of pre-immune serum
Day 14	Second immunisation (1 st booster injection) with antigen preparation, sampling of serum
Day 28	Third immunisation (2 nd booster injection) with antigen preparation, sampling of serum
Day 38	Final bleeding

3.6.7 Purification of antisera

In order to reduce background signals, antisera directed against GST fusion proteins were purified by adsorption to whole cell lysates of the *E. coli* carrying the pGEX5x3 vector and the respective deletion mutant. A 100 ml culture of *E. coli* HB 101 carrying the pGEX5x3 vector was grown to an OD₆₀₀ of 0.3 to 0.4 in the appropriate medium, then the culture was induced with IPTG (1 mM) and incubated for 2 hours before centrifugation. The cells were resuspended in 500 µl A. dest. and 500 µl SDS splitting buffer. The samples were heated to 100 °C for 3 min and then SDS PAGE and Western blotting was performed as described above. Nitrocellulose membranes with a 35 cm² surface with the bound bacteria were used for adsorption of 10 ml serum (1:10 dilution in 0.85 % NaCl) over night at room temperature on a rolling incubator. A second adsorption was accomplished as described above with the respective deletion mutant strain for the anti-GST-Ape51 serum or the anti-GST-FrpB serum. Serum directed against *A. pleuropneumoniae* wt outer membrane preparation and serum of pigs reconvalescent from *A. pleuropneumoniae* infection were adsorbed against the respective deletion mutant: Bacteria were grown over night under the

appropriate conditions in a liquid culture and harvested by centrifugation. The cells were resuspended in 1 ml 10 % formaldehyde for inactivation over night. The bacteria were washed twice with 1 ml A. dest. and pelleted via centrifugation again. The pellet was resuspended in 10 ml (1:10 dilution in 0.85 % NaCl) of antiserum which was incubated on a rolling shaker at room temperature over night.

If necessary the adsorption of all sera was repeated for further reduction of background signals. In order to reduce further cross-reactivity with remaining IgM antibodies, the adsorbed sera were purified over protein G columns¹ according to the manufacturer's instructions.

3.7 Virulence studies

An aerosol infection model was used to test for the virulence of the *A. pleuropneumoniae ape51* mutant and the GFP-labelled mutant. The experimental set up was chosen to mimic the natural route of infection in pigs 7 to 8 weeks of age.

Timeline of challenge experiments

Day -7:	Arrival at the facility, blood samples taken for enzyme linked immunosorbent assay (ELISA)
Day -1:	Clinical examination (including determination of body temperature)
Day 0:	Clinical examination (including determination of body temperature), aerosol infection
Days 1-7:	Clinical examination (including determination of body temperature)
Day 7:	Blood samples taken; euthanasia and post mortem examination of some animals
Day 21:	Blood samples taken; euthanasia and post mortem examination of all remaining animals

For infection with the green fluorescent protein *A. pleuropneumoniae* GFP marker strain a different timeline applies:

¹ Protein G HP SpinTrap™, GE Healthcare, Freiburg, Germany

- Day -7: Arrival at the facility, blood samples taken for enzyme linked immuno sorbent assay (ELISA)
- Day -1: Clinical examination (including determination of body temperature)
- Day 0: Clinical examination (including determination of body temperature), aerosol infection
- Day 1: Clinical examination (including determination of body temperature), euthanasia and post mortem examination

3.7.1 Origin and housing of the animals

Pigs (German Landrace) were purchased from the Züchtungszentrale Deutsches Hybridschwein¹ at 6 to 7 weeks of age. The animals originated from an *A. pleuropneumoniae*-free herd with no clinical symptoms and no serological response to an ApxII-ELISA (LEINER et al. 1999) prior to infection. All animals were treated and cared for in accordance with the principles outlined in the “European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes” (European Treaty Series, No. 123 [<http://conventions.coe.int/Treaty/EN/Treaties/Html/123.htm>; ETS No. 170]). Pigs were randomly assigned to two different groups and kept in separately ventilated isolation units.

For infection with the *A. pleuropneumoniae* GFP marker strain the animals were housed under S2- conditions in the Institute of Virology, University of Veterinary Medicine Hannover, Germany.

3.7.2 Aerosol infection chamber

The animals were infected in an aerosol infection chamber built by the Impfstoffwerk Dessau-Tornau². The infections were carried out according to descriptions of JACOBSEN et al. (1996). It is possible to simultaneously infect up to 6 pigs, 7 to 8

¹ Dahlenburg, Germany

² Dessau, Germany

weeks of age. Surveillance of the animals during aerosol exposure is facilitated through the top of the chamber which consists of an acrylic window. The air is exchanged through two vents equipped with filters, one of which is connected to a compressor¹. The bacterial suspension is aerosolised via a nozzle² operated by compressed air³. All tubing is either made of autoclaveable silicone or Teflon®.

3.7.3 Preparation of bacteria for aerosolisation

For aerosol infection, a 90 ml culture was inoculated with 10 ml of an overnight liquid culture of the *A. pleuropneumoniae* strain under investigation and grown with shaking at 37°C to an OD₆₀₀ of 0.4 to 0.5. The culture was placed on ice for 10 minutes, diluted 1:300 in ice-cold NaCl (0.85 %), and kept on ice until use for a maximum of 2 h. Immediately prior to aerosolisation, bacteria were further diluted 1:100 in ice-cold NaCl (0.85 %) resulting in approximately 1×10^5 colony forming units (cfu) per 13 ml dose (for four pigs); upon aerosolisation, this dose corresponds to approximately 1×10^2 *A. pleuropneumoniae* cells per litre of aerosol in the chamber, a dose which had been titrated for the *A. pleuropneumoniae* strain AP76 to induce severe but not fatal disease (Teutenberg-Riedel et al., unpublished data). For aerosolisation of the GFP-labelled *A. pleuropneumoniae* the bacteria were diluted 1:10 resulting in approximately 9.5×10^5 cfu per 13 ml dose (for 2 pigs) or the 1:300 dilution was directly used for infection resulting in 4.7×10^7 cfu per 13 ml dose (for 2 pigs).

3.7.4 Aerosol infection

Groups of four pigs at a time were challenged in the chamber, for the *A. pleuropneumoniae* GFP marker strain two pigs were challenged together. To achieve an even distribution of the aerosol in the chamber, the nozzle was fixed at a setting of "5" and the valve regulating the flow of the fluid was set to "75". The challenge dose, 13 ml of diluted bacterial suspension, was aerosolised at a pressure

¹ KNF Neuberger, Freiburg, Germany

² Model no. 97058, Schlick Duesen, Untersiemau, Germany

³ Linde, Hannover, Germany

of 2 bar within a time of approximately two minutes. Ten minutes after completing aerosolisation, the air in the chamber was exchanged ten times over the duration of 20 min using a compressor before the pigs were led back to their stable.

3.7.5 Surveillance of the animals during the experiment

Body temperature and clinical symptoms were recorded at least daily for each individual pig or as needed. A clinical scoring system based on the directive in the European Pharmacopoeia for testing *A. pleuropneumoniae* vaccines (Porcine Actinobacillosis Vaccine [Inactivated]; 3rd edn, EDQM, Council of Europe, Strasbourg, France) was employed to assess the clinical condition of each individual animal as follows: A score of one was given for each, the occurrence of coughing, dyspnea and vomitus, resulting in a minimum clinical score of zero and a maximum score of three per day; the added daily clinical scores of days 1 to 7 were designated as the total clinical score. Statistical analysis of the total clinical score was performed using the Student's T-Test.

3.7.6 Post mortem examination

Pigs were euthanised on day 1, 7 or 21 post infection (depending on the experimental setup) by intravenous injection of 5-10 ml of Eutha 77[®]¹ per pig.

3.7.6.1 Determination of lung lesion scores

The lung damage caused by *A. pleuropneumoniae* infection under laboratory conditions was assessed by the scheme of HANNAN et al. (1982). Individual lung lesions are mapped on a simplified diagram of the porcine lung in which each lobe is subdivided into triangles. The damage of each lobe is evaluated resulting in a score between 0 (= healthy) and 5 (= completely pathologically altered). By separate scoring of each of the 7 lobes a maximum score of 35 for entire lung can be obtained. As the lung lobes were subdivided into triangles and the number of

¹ Pentobarbital, Essex Pharma, Munich, Germany

'affected' triangles is counted, the score for this lung lobe is calculated as a fraction of five (HANNAN et al. 1982). This scheme has been adopted in the European Pharmacopoeia¹ (3rd edn, EDQM, Council of Europe, Strasbourg, France) as the reference method in vaccine trials for *A. pleuropneumoniae*. Statistical analysis of the total clinical score was performed using the Wilcoxon Signed-Rank test.

3.7.6.2 Bacteriological examination of organ samples

The bacteriological examination included organ samples of palatine tonsils, and bronchial lymph nodes and of defined positions located in the outer third of each of the seven lung lobes; an additional sample of affected lung tissue was taken, if it was not covered by any of the defined organ samples. Organs were plated on Columbia sheep blood agar (CSB) to exclude other bacterial infections as well as on selective blood agar (JACOBSEN and NIELSEN 1995) by directly touching the agar with the organ sample and fractionating twice. A score for reisolation of 0 was given if *A. pleuropneumoniae* colonies grew only in the directly touched area and a score of 1 was given if colonies were found in the fractionated streaks. For the reisolation score these numbers were added up for each pig in the respective group, and the arithmetic mean and standard deviation were determined. Several individual *A. pleuropneumoniae*-like colonies were subcultured on supplemented PPLO agar and confirmed by urease assay and PCR analyses using primers oApe-1 and oApe-4 to distinguish the *ape51* mutant from the wild type *A. pleuropneumoniae*; and primers oTbpB3G and oTbpA4G for confirmation of the *A. pleuropneumoniae* GFP marker strain.

3.7.7 Histopathological examination of lung tissue from infected animals

For histopathological examination affected and unaffected lung was collected from animals infected with the *A. pleuropneumoniae frpB* mutant and the wild type-infected control group. Lung tissue was fixed in 10 % formaldehyde overnight.

¹ <http://www.pheur.org>

Afterwards it was embedded in paraffin wax and cut into slices 4 to 5 µm in thickness. The slices were then stained in hematoxilin and eosin.

3.7.8 Enzyme Linked Immunosorbent Assay (ELISA)

The humoral immune response of pigs was tested in a standardised ELISA with the recombinant *A. pleuropneumoniae* ApxIIA protein as solid phase antigen (LEINER et al. 1999). Antibody levels against the protein were assessed to monitor the animals' previous contact with *A. pleuropneumoniae*.

3.8 Fluorescence detection

3.8.1 Fluorescent activated cell sorting (FACS)

Flow cytometry was performed using the FACSCalibur system¹. Samples were analysed for scattering properties (SSC: sideward scattering) and green fluorescence (channel FL-1, measurement of green fluorescence and autofluorescence at a wavelength of 515-545nm).

3.8.1.1 Preparation of samples

For preparation of samples for FACS analyses 3 ml of phosphate buffered saline (PBS) + 0.1 % Triton-X 100 were added to 2.5 g lung tissue. Samples were thoroughly homogenised using an ULTRA-TURRAX². Then the homogenate was diluted with 10 ml PBS + 0.1 % Triton-X 100. In order to remove particles too large for FACS analysis, samples were sieved through a filter with a pore size of 125 µm. The mixture was further diluted 1:100 with PBS + 0.1 % Triton-X 100 and 0.5 % formaldehyde to obtain a number of particles suitable for FACS analysis and for inactivation of the bacteria. Liquid cultures were induced with Na₃CaDTPA (300 µM) to enhance fluorescence and then diluted 1:100 with PBS + 0.1 % Triton-X 100 and 0.5 % formaldehyde.

¹ Beckton Dickinson, San Jose, USA

² IKA® Werke, Staufen, Germany

PBS 8.0 g NaCl
0.2 g KCl
1.43 g Na₂HPO₄ X 2 H₂O
0.2 g KH₂PO₄
Ad 1 litre Aqua dest.
pH was adjusted to 7.0 with HCl

3.8.2 Fluorescence detection with TECAN-GENios Pro fluorescence reader

Further measurements of fluorescence were done in a plate-based system with the TECAN-GENios Pro[®] fluorescence reader¹. Green fluorescence was measured at an emission wavelength of 535 nm in black 96-well-plates² to inhibit modification of the measurements by reflections of light. In liquid cultures of the *A. pleuropneumoniae* GFP marker strain, iron restriction was induced with Na₃CaDTPA (300 µM) to enhance fluorescence.

3.8.3 Fluorescence microscopy

Fluorescence microscopy was accomplished with the Leica DM LB fluorescent microscope³. In liquid cultures of the *A. pleuropneumoniae* GFP marker strain, iron restriction was induced with Na₃CaDTPA (300 µM) to enhance fluorescence.

¹ Tecan, Männedorf, Switzerland
² Greiner, Frickenhausen, Germany
³ Leica Microsystems, Wetzlar, Germany

4 Results

4.1 In-vivo tracing of an *A. pleuropneumoniae* GFP marker strain

An important factor influencing the environment of *A. pleuropneumoniae* in the host is iron limitation. The effects of limited iron have been investigated in vitro. However, little is known about host-induced changes of transcription in *A. pleuropneumoniae* and its actual location in vivo. In order to try and investigate this issue, a GFP marker strain was constructed and used in an aerosol infection experiment. The study objectives were to test the virulence of the marker strain and to reisolate it from the pig's lower respiratory tract. It was of particular interest whether the intensity of GFP-expression would be sufficiently strong to perform a quantitative reisolation by FACS analysis and to determine the location of individual bacteria in the tissue by fluorescence microscopy.

4.1.1 Construction of an *A. pleuropneumoniae* GFP-marker strain

For construction of a GFP-marker strain the single step transconjugation system was used (OSWALD et al. 1999). The constructed marker strain carries the *gfpmut3* gene inserted between the genes encoding for the transferrin binding proteins TbpB and TbpA resulting in an artificial operon without an antibiotic resistance marker (Fig.1). With this construct it is possible to induce fluorescence by limitation of available iron as transcription of the *tbp* operon is induced when iron is scarce.

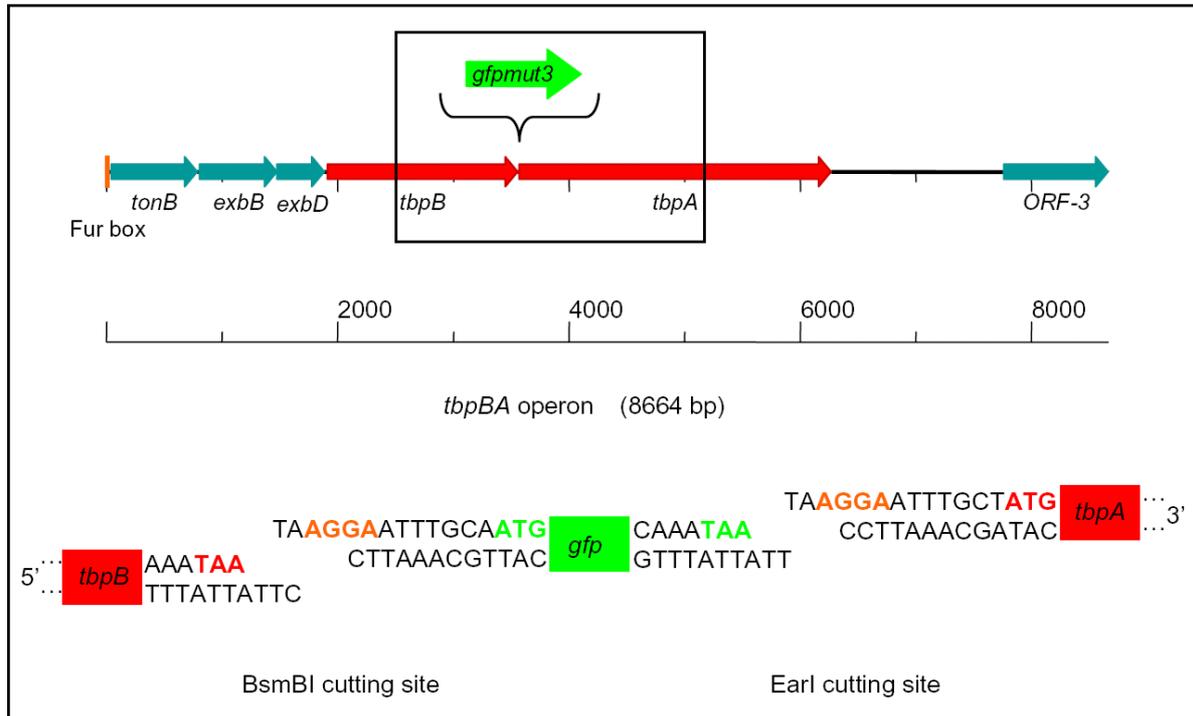


Fig.1 Schematic of the *tbpBA-gfp* operon of *A. pleuropneumoniae* marker strain. The *gfpmut3* gene was inserted between the *tbpB* and *tbpA* genes. In the operon the transferrin binding proteins are preceded by the *tonB*, *exbB* and *exbD* genes encoding for energy coupling proteins. The operon is regulated by Fur (Ferric uptake regulator). The boxed area is enlarged below with the respective restriction endonuclease cutting sites. Start and stop codons and the Shine-Dalgarno sequences are highlighted. Arrows indicate the orientation and relative length of the respective open reading frames. Numbers indicate the size in basepairs (bp).

4.1.1.1 Construction of pTGT 700 for insertion of the *gfp* gene between the *tbpB* and the *tbpA* genes

The *tbpB* gene was amplified from chromosomal DNA of *A. pleuropneumoniae* AP76 with primers oTbpB1G and oTbpB2G, and the resulting PCR product was cloned into pCR[®]2.1-TOPO resulting in plasmid pTB801. The *tbpA* gene was also amplified from chromosomal DNA of *A. pleuropneumoniae* AP76 by PCR using primers oTbpA1G and oTbpA2G. The *gfpmut3* gene was obtained from plasmid pMW102 by amplification with primers oGFPI1 and oGFPI2. The *gfp* and the *tbpA* PCR products were digested with Earl and ligated. The *tbpA::gfp* ligation fragment was cloned into pCR[®]2.1-TOPO resulting in plasmid pGFPA801. In order to complete the construct, plasmid pGFPA801 was digested with BsmBI and Apal; plasmid pTB801 was cut

with the same enzymes to release the *tbpB*-containing fragment which was then ligated into pGFPA801 resulting in plasmid pTGT801. For construction of the transconjugation plasmid pTGT700, both plasmid pTGT801 and the transconjugation vector pEMOC2 were digested with *Apal* and *NotI*, and the *tbpB-gfp-tbpA*-fragment was ligated into pEMOC2 (Fig. 2). The correct localisation of *gfp* was confirmed by PCR, restriction enzyme digest, and nucleotide sequence analysis.

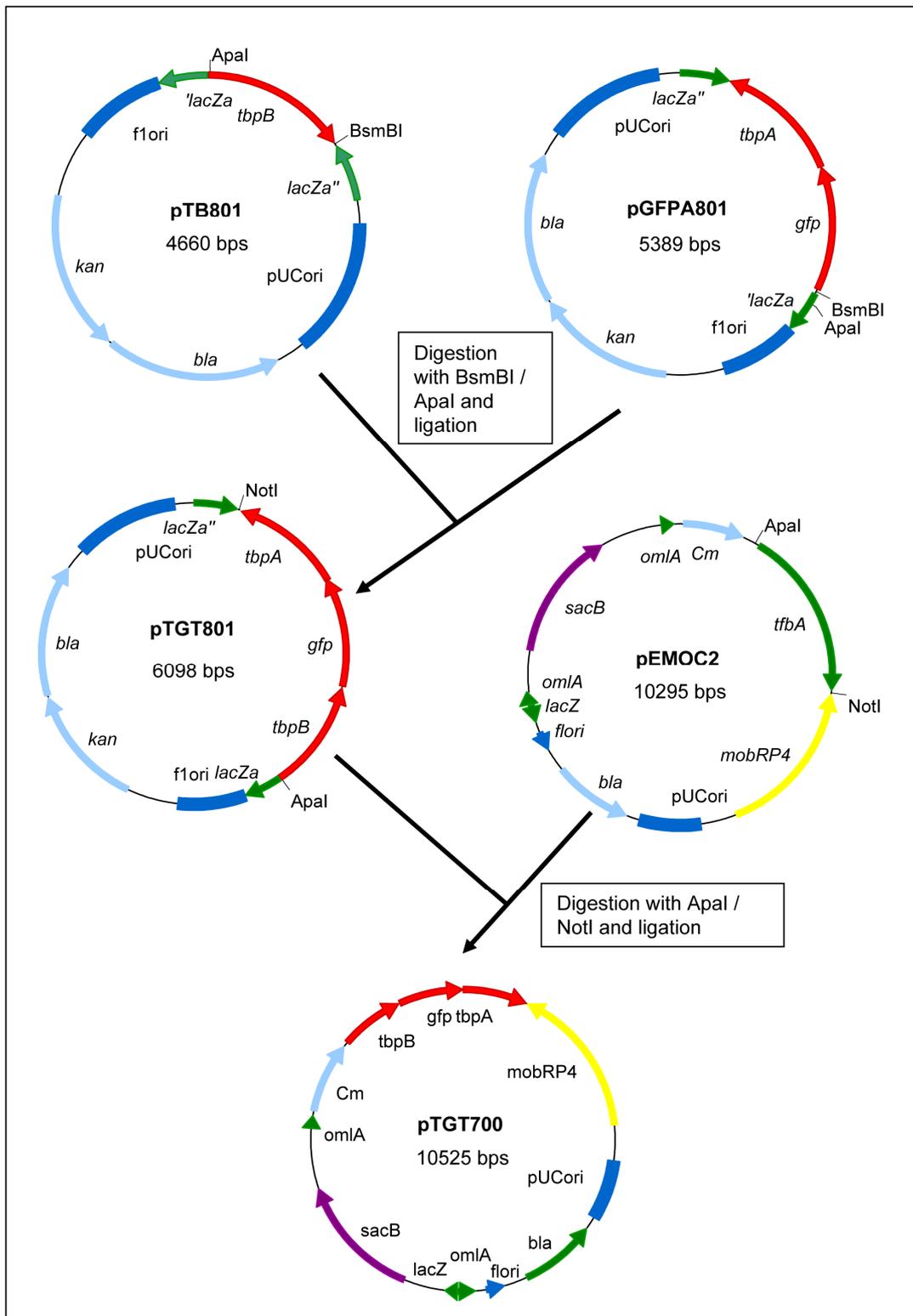


Fig. 2 Construction of plasmid pTGT 700. Arrows on the circular maps indicate the respective reading frames. *bla* beta-lactam antibiotics resistance determinant, *kan* kanamycin resistance determinant, *Cm* chloramphenicol resistance determinant, *sacB* levansucrase of *B. subtilis*, the counterselectable marker, insertion of the *tbpB-gfp-tbpA*-operon is highlighted in red.

4.1.1.2 Construction of the *A. pleuropneumoniae* GFP marker strain

For construction of the *A. pleuropneumoniae* GFP marker strain the conjugative plasmid pTGT700 was transferred into *A. pleuropneumoniae* AP76 by conjugation using *E. coli* β 2155 as the donor strain. Colonies resistant to chloramphenicol were selected, and cointegration was confirmed using PCR analyses. Individual colonies with the correct PCR profile underwent the counterselection procedure. Colonies obtained after counterselection were confirmed by PCR analysis with primers oTbpB3G and oTbpA4G. Functionality of the *tbpB::gfp::tbpA*-construct was confirmed by fluorescence microscopy and the maintained ability to use transferrin in a plate bioassay (Fig. 3).

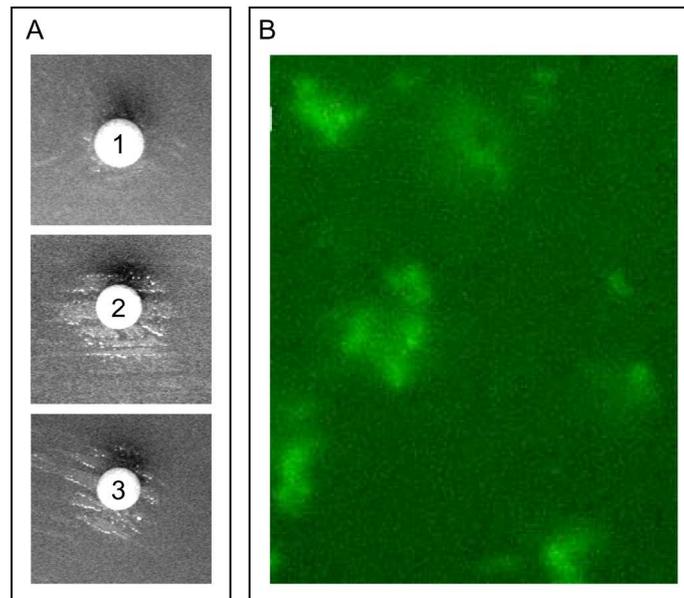


Fig. 3 Functionality of the *A. pleuropneumoniae* GFP marker strain. A) *A. pleuropneumoniae* GFP marker strain grown on BHI agar with 1) buffer (negative control), 2) ferric citrate (positive control) and 3) porcine transferrin; B) Fluorescent microscopy of a liquid culture of the marker strain.

4.1.2 In vivo application of the *A. pleuropneumoniae* GFP marker strain

4.1.2.1 Aerosol infection experiments, clinical observations, and post mortem analysis

In an aerosol infection experiment four pigs were infected with the *A. pleuropneumoniae* GFP marker strain by aerosolisation 4.7×10^7 cfu (group 1; two pigs), and 9.5×10^5 cfu (group 2; two pigs). All animals were sacrificed on day one after infection due to severe clinical symptoms. The animals showed severe dyspnoea and coughing. A rise of temperature above 40°C was observed in 3 pigs, one animal already had a subnormal temperature of 35.4°C. These results show that the *A. pleuropneumoniae* GFP marker strain is still virulent.

At necropsy, the lungs of all pigs showed multiple haemorrhages with a diameter of 5 to 20mm. It was noticeable that the whole thoracic cavity was filled with fibrin. The lung surface was also covered with fibrin in all animals examined. The *A. pleuropneumoniae* GFP marker strain could be isolated from each lung lobe in the infected pigs.

4.1.2.2 Analysis of fluorescence activity

Liquid cultures of reisolated bacteria of the *A. pleuropneumoniae* GFP marker strain obtained at post mortem analysis were investigated by fluorescence microscopy (Fig. 4).

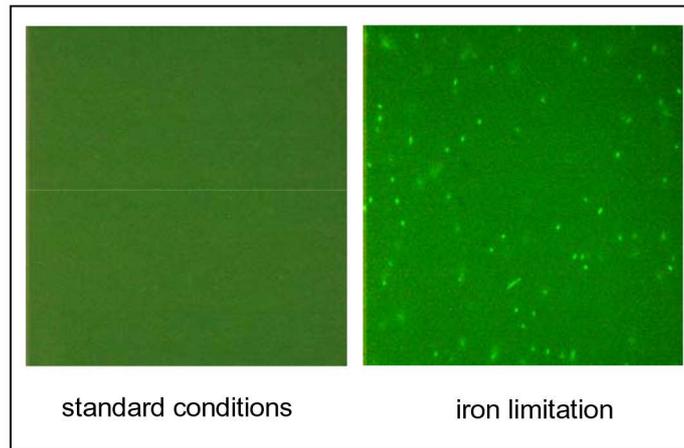


Fig. 4 Fluorescence microscopy. The *A. pleuropneumoniae* GFP marker strain reisolated at post mortem analysis; growth in liquid medium under standard and iron-restricted conditions.

In order to test whether individual bacteria of the *A. pleuropneumoniae* GFP marker strain could also be detected directly from tissue by FACS analysis, the following samples were analysed by flow cytometry: A) homogenate of infected lung tissue, B) homogenate of healthy lung tissue (control for organ-specific autofluorescence), C) *A. pleuropneumoniae* reisolated from the infected animals grown in liquid culture and induced for GFP-expression by iron-deficiency (positive control) and D) *A. pleuropneumoniae* wt in liquid culture (negative control). It was not possible to distinguish the GFP-labelled bacteria from lung tissue homogenate as the lung's autofluorescence was strongly interfering with the bacterial fluorescence. Nevertheless, it was possible to detect fluorescence in liquid cultures of bacteria reisolated from infected lungs thereby confirming the results of fluorescence microscopy and showing that the insertion of the *A. pleuropneumoniae* GFP marker strain is stable under in vivo conditions (Fig. 5).

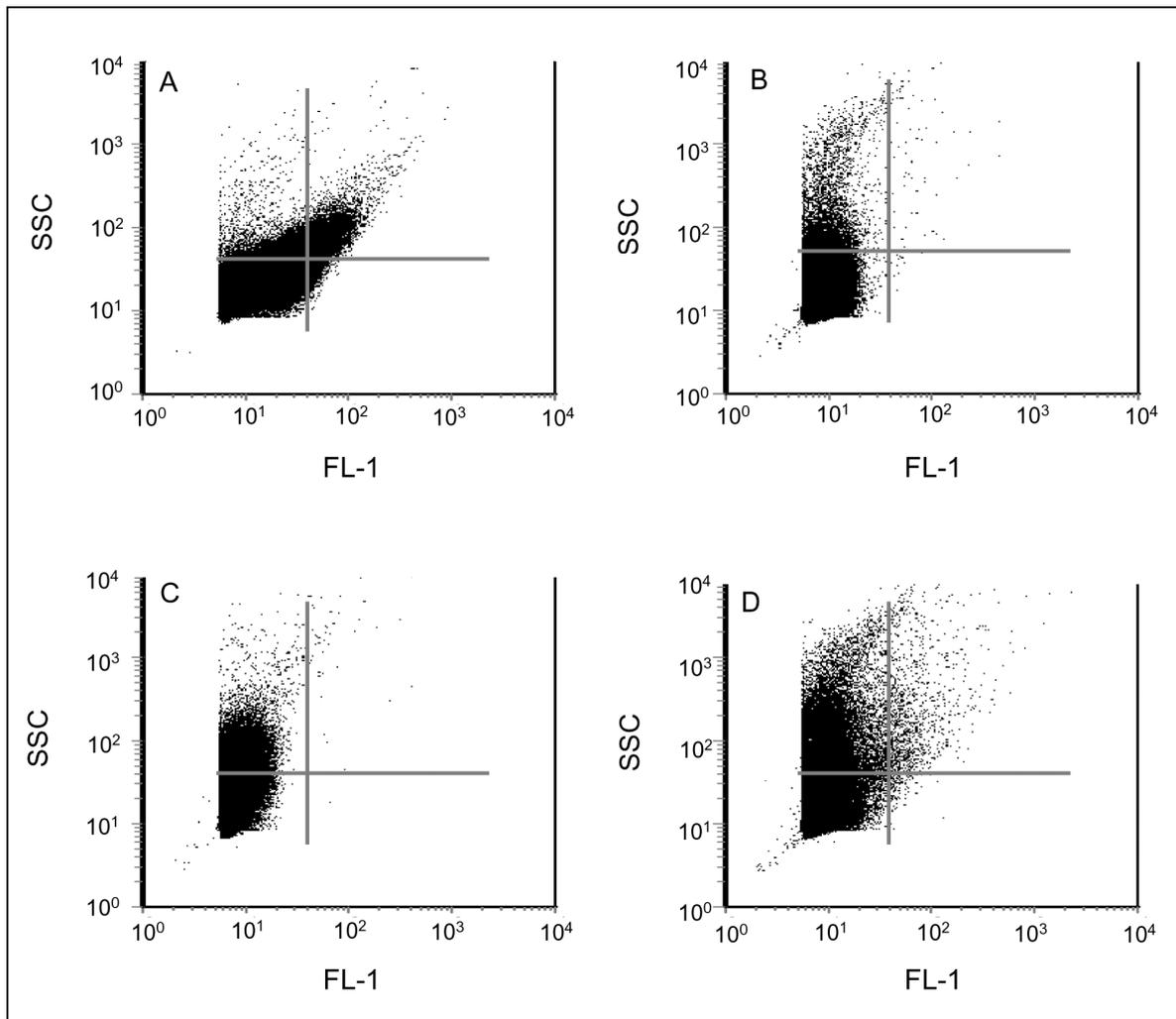


Fig. 5 FACS analyses of the *A. pleuropneumoniae* GFP marker strain. A) *A. pleuropneumoniae* GFP marker strain reisolated from infected lung tissue and cultured in liquid medium with iron limitation; fluorescent bacteria are demonstrated in the upper right quadrant; B) Lung homogenate of an animal infected with the *A. pleuropneumoniae* GFP marker strain; C) *A. pleuropneumoniae* wt cultured in liquid medium with iron limitation, D) Lung homogenate of a healthy animal; SSC) sideward scatter, FL-1) total fluorescence (GFP fluorescence and autofluorescence).

Since FACS analysis did not allow the detection and quantification of the *A. pleuropneumoniae* GFP marker strain directly from lung tissue, fluorescence detected with the TECAN GENios Pro fluorescence reader was also investigated. The obtained results confirmed the results from FACS analyses. The fluorescence from non-infected lung homogenate and infected lung homogenate were indistinguishable in the magnitude of fluorescence (data not shown) whereas fluorescence of the cultured *A. pleuropneumoniae* GFP marker strain during iron

starvation is increased by more than 4-fold in comparison to culture conditions without iron starvation. This increase in fluorescence of the *A. pleuropneumoniae* GFP marker strain is highly significant ($p = 0.0001$). Fluorescence of *A. pleuropneumoniae* wt, serving as a negative control was on the same level as the non-induced *A. pleuropneumoniae* GFP marker strain (Fig. 6).

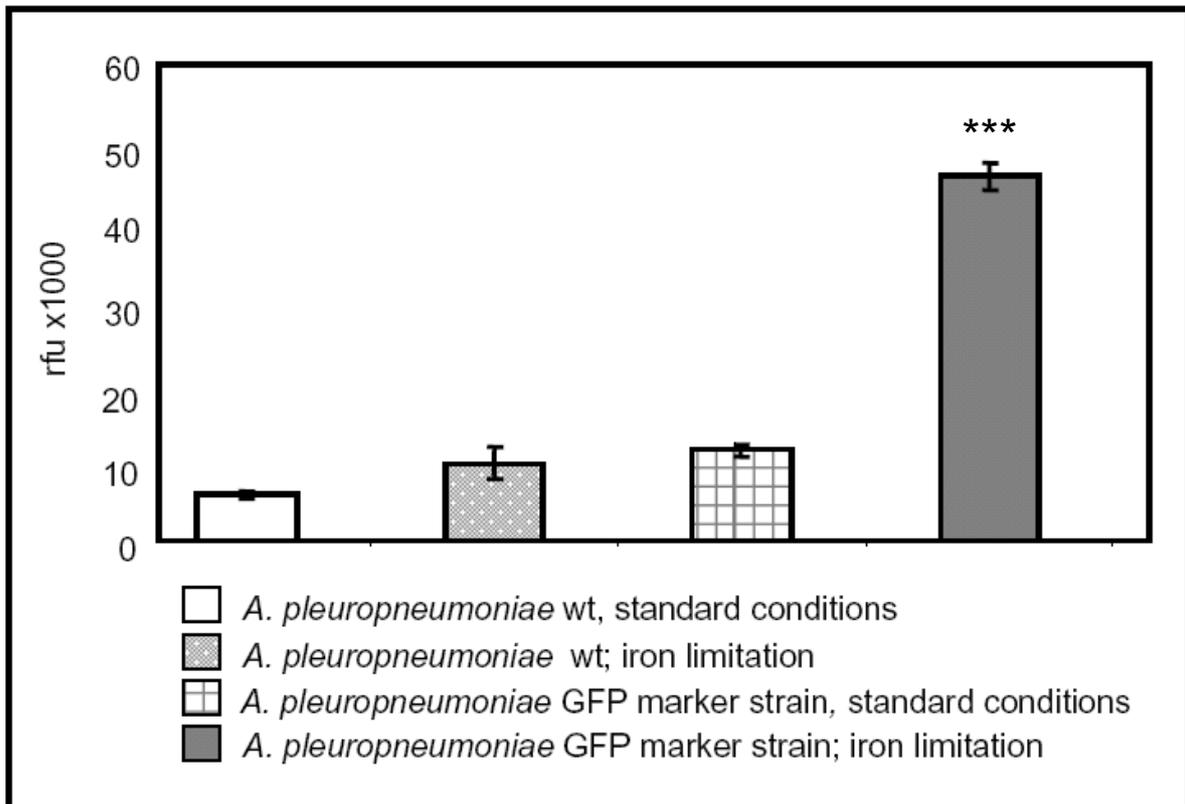


Fig. 6 Measurement of fluorescence in *A. pleuropneumoniae* liquid culture. Bars represent the arithmetic means, hinges the standard deviation as determined from three independent measurements. Fluorescence is measured in relative fluorescent units (rfu). Statistical analysis was performed using Student's t-test. Asterisks denote statistical significance ($p < 0.001$).

Since the *A. pleuropneumoniae* GFP marker strain could neither be detected directly by FACS analysis nor in the TECAN reader, it was investigated whether the fluorescence of the GFP marker strain was sufficient for direct detection by fluorescent microscopy in infected lung tissue. It was found that individual bacteria could not be detected due to strong autofluorescence of lung tissue (data not shown). Furthermore indirect detection of GFP via a commercial monoclonal antibody was

tried to detect the GFP marker strain lysed bacteria in a Western blot approach. Unfortunately, the antibody failed to recognise the GFP labelled *A. pleuropneumoniae* (data not shown) and in vivo detection in immunohistochemistry with this antibody was not further pursued.

Since the *A. pleuropneumoniae* GFP marker strain could not be shown to be feasible for in vivo studies, alternative approaches were to be tested. Proteins regulated by either one of the two main anaerobic regulators ArcA and HlyX were chosen to monitor protein expression and to further characterise them. The iron-regulated outer membrane protein B (FrpB) was chosen as a candidate to monitor HlyX regulation while Ape51 was chosen to monitor the effects of ArcA regulation as both candidates were shown to be solely influenced by one of the regulators (BUETTNER 2008).

4.2 The iron-regulated outer membrane protein B (FrpB) of *A. pleuropneumoniae*

The FrpB protein is strongly regulated by HlyX (8.8 fold upregulation) and, therefore, this protein could potentially serve as a marker for HlyX activity. FrpB was shown to be an integral outer membrane protein, and it was demonstrated that an isogenic mutant lacking the *frpB* gene was completely avirulent (BENDALLAH 2008). As the reason for this loss of virulence had not been elucidated, the in vitro phenotype of the mutant as well as the function of the FrpB protein were to be determined prior to using this protein as a marker for HlyX-activity in vivo.

4.2.1 Growth and survival characteristics of *A. pleuropneumoniae* Δ *frpB*

Growth curves of *A. pleuropneumoniae* wt and *A. pleuropneumoniae* Δ *frpB* were performed under anaerobic conditions. Cultures were monitored for 24 to 30 hours displaying comparable growth characteristics in both strains. Likewise, survival kinetics were indistinguishable under anaerobic conditions (Fig. 7).

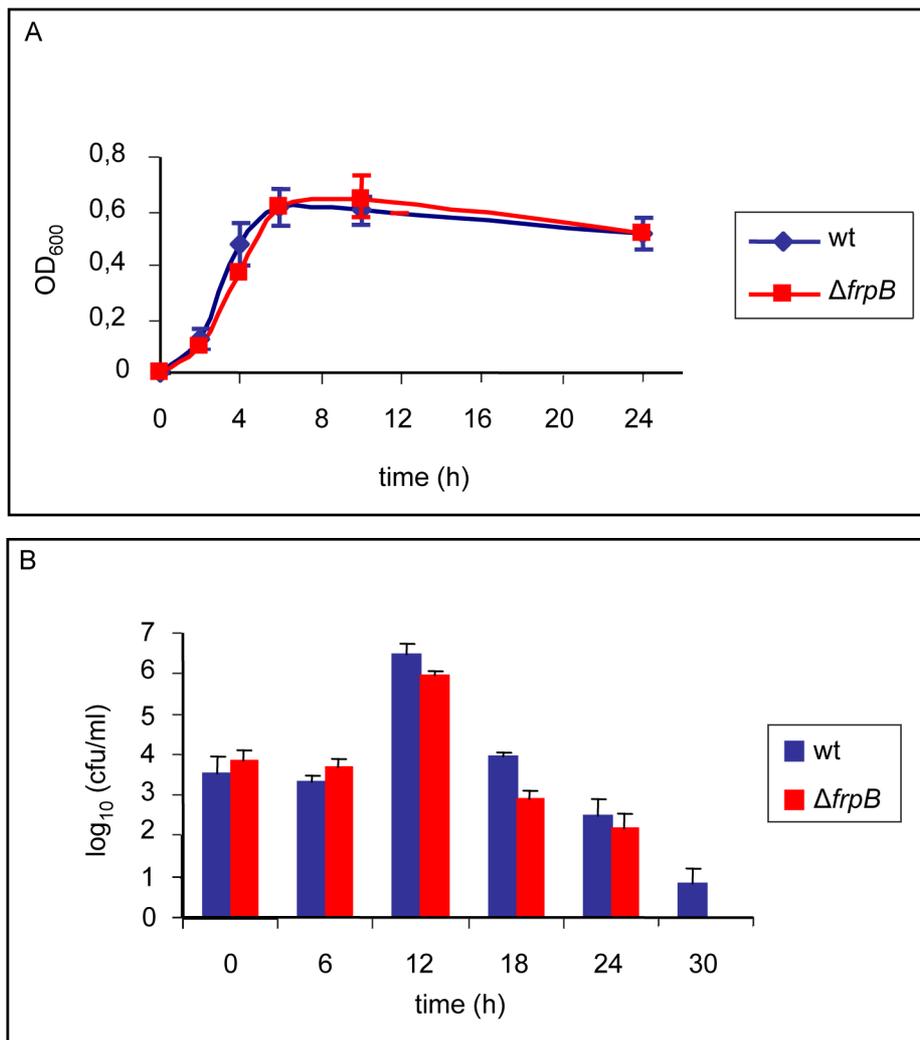


Fig. 7 Anaerobic growth and survival of *A. pleuropneumoniae* wt and $\Delta frpB$. A) Growth characteristics under anaerobic conditions; B) Survival characteristics under anaerobic conditions. Differences in growth and survival of both strains are statistically insignificant.

4.2.2 Regulation of the FrpB protein by the ferric uptake regulator (Fur)

The annotation of FrpB as iron-regulated protein B implies that expression of the protein might be regulated by iron. Since Fur (ferric uptake regulation protein) is known to be the major regulator for iron-dependent proteins, it was hypothesised that expression of the FrpB of *A. pleuropneumoniae* is controlled by Fur in addition to HlyX. Sequence alignment of the upstream sequence gave no significant agreement to a Fur box consensus sequence (the best hit 81 bp upstream of the *frpB* start codon showed a sequence identity of 10 out of 19 bases in comparison to the *E. coli*

consensus sequence). To investigate whether a possible indirect regulation by Fur occurs, three independent biological repeats of outer membrane preparations of *A. pleuropneumoniae* wt and *A. pleuropneumoniae* Δfur grown to late log phase were prepared, run on SDS gels, and lane intensities were compared semiquantitatively. It could be shown that the FrpB protein is upregulated 2.1-fold in the *fur* deletion mutant. Although the FrpB protein is only slightly upregulated in *A. pleuropneumoniae* Δfur this result indicates that expression of the *frpB* gene, in addition to HlyX-regulation, could be indirectly regulated by Fur when iron availability is limited (Fig. 8).

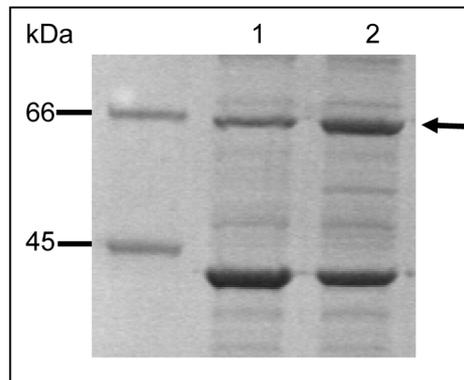


Fig. 8 Comparison of outer membrane preparations of *A. pleuropneumoniae* wt and Δfur . 1) *A. pleuropneumoniae* wt, 2) *A. pleuropneumoniae* Δfur , the arrow indicates the position of the FrpB protein, numbers to the left indicate the size in kDa.

4.2.3 Plate bioassay

In order to investigate the possible function of FrpB the ability of *A. pleuropneumoniae* $\Delta frpB$ to use different sources of iron was analysed in a plate bioassay. It could be shown that *A. pleuropneumoniae* $\Delta frpB$ was able to grow with ferric citrate as sole iron source but not with porcine transferrin. Complementation of *A. pleuropneumoniae* $\Delta frpB$ in trans restored the ability to utilise porcine transferrin, while transformation of the mutant with the vector pLS88 did not restore the phenotype. *A. pleuropneumoniae* wt functioned as a positive control for the ability to grow with different iron sources, *A. pleuropneumoniae* $\Delta tbpBA$ was used as a negative control with respect to the use of porcine transferrin (Fig. 9).

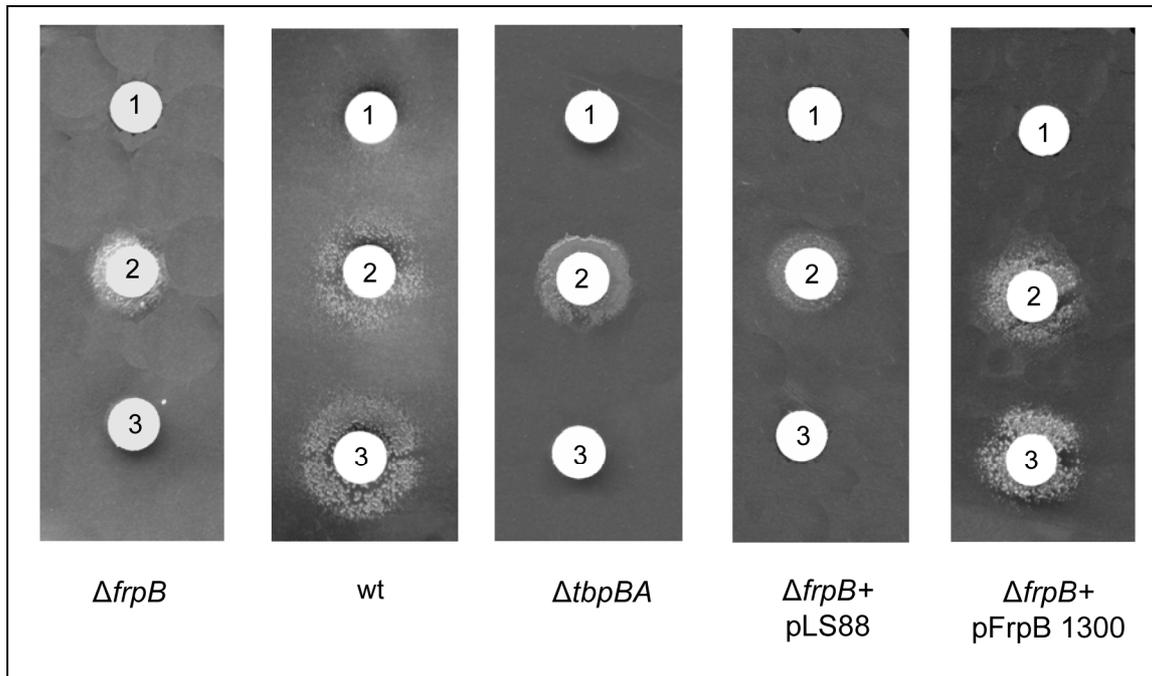


Fig. 9 Plate bioassay. *A. pleuropneumoniae* $\Delta frpB$, *A. pleuropneumoniae* wt (positive control), *A. pleuropneumoniae* $\Delta tbpBA$ (negative control), *A. pleuropneumoniae* $\Delta frpB + pLS88$ and *A. pleuropneumoniae* $\Delta frpB + pFrpB1300$, grown on BHI agar with 1) buffer (negative control), 2) ferric citrate and 3) porcine transferrin as iron sources.

4.2.4 Histopathological examination of lung tissue from infected pigs

A deletion of the *frpB* gene has been shown to render *A. pleuropneumoniae* avirulent with the pigs not developing a humoral immune response and with no reisolation of *A. pleuropneumoniae* one week after infection (BENDALLAH 2008). In this work it was investigated whether any histopathological changes in lung tissue might have occurred during infection.

It was shown that on day 7 and on 21 p. i. animals in the *A. pleuropneumoniae* $\Delta frpB$ -infected group had a mild pleuritis pulmonalis and a mild interstitial pneumonia. *A. pleuropneumoniae* wt infected animals showed a moderate to severe pleuritis pulmonalis, an interstitial pneumonia with atelectases and focal fibrosis. A catarrhalic-purulent bronchopneumonia was also found (Fig. 10). Furthermore examination of the local lung lymph nodes revealed a severe follicular hyperplasia in the wt group whereas the mutant group showed no activation.

These findings underline the previous results and support the complete avirulence of *A. pleuropneumoniae* Δ *frpB* since it is neither inducing histopathological alterations in the porcine lung nor does it activate the local lymph nodes.

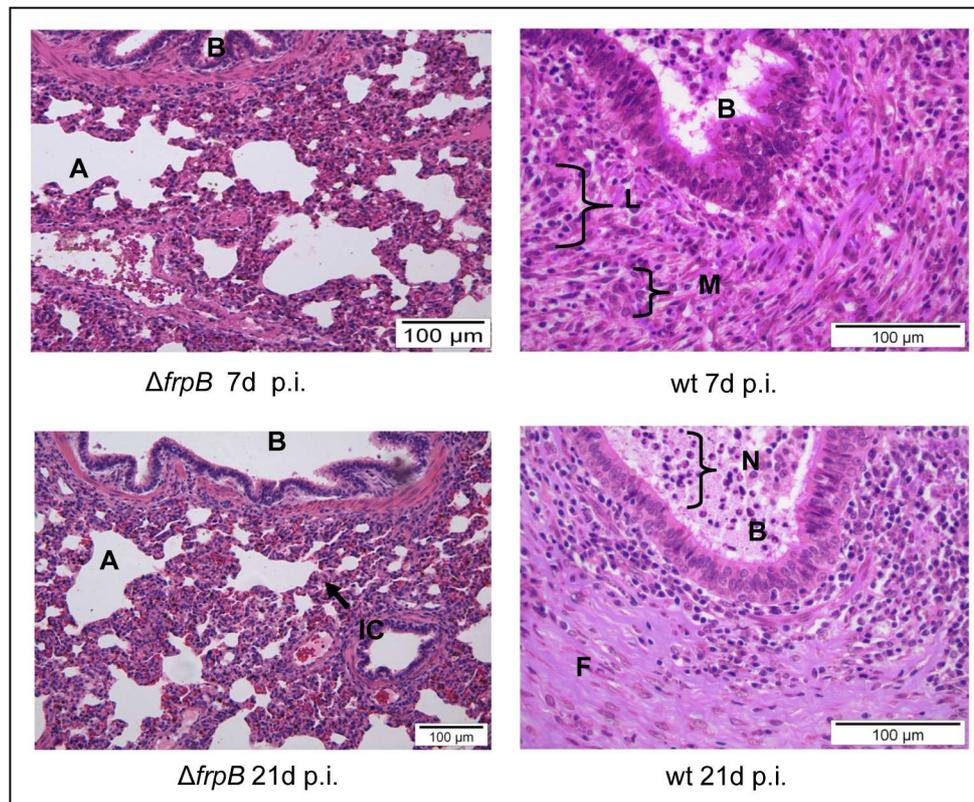


Fig. 10 Histopathology of lung lesions stained with hematoxylin and eosin. A) alveole; B) bronchus; F) fibrin; IC) interstitial consolidation; L) lymphocytes; M) macrophages; N) neutrophilic granulocytes. The *A. pleuropneumoniae* wt-infected animals showed lung alterations typical to Porcine Pleuropneumonia while the *A. pleuropneumoniae* Δ *frpB*-infected animals only showed a mild interstitial consolidation.

4.2.5 Detection of the FrpB protein with polyclonal anti serum

Since the FrpB protein – despite its function in iron-uptake – is mostly regulated by HlyX it was considered to be a suitable target to investigate the expression of HlyX-regulated proteins in vivo. Therefore, part of the *frpB* gene was cloned into an expression vector facilitating inducible expression of a GST-FrpB fusion protein with a total size of 83 kDa. Polyclonal antibodies were raised against both the GST-FrpB fusion protein as well as against outer membranes of *A. pleuropneumoniae* wt. This

approach was considered to result in antibodies specifically detecting linear epitopes of the denatured protein as well as conformational epitopes present in the native protein localised in the outer membrane. These antibodies were required to be able to semiquantitatively determine the protein in Western blots as well as in formaldehyde-fixed lung tissue slices of infected pigs. Purification of the antibodies was done via adsorption against denatured whole cell lysates of *E. coli* HB101 carrying plasmid pGEX5x3 and against denatured whole cell lysates of *A. pleuropneumoniae* Δ *frpB* for the serum raised against the GST-fusion protein. The serum against the membrane preparation was adsorbed against formaldehyde-fixed bacteria for purification. Adsorptions were done to reduce unspecific background signals. In addition, both sera were further purified with a protein G column.

The serum raised against the GST-FrpB fusion protein recognised the GST-FrpB fusion protein as well as the native FrpB protein in Western Blot analysis confirming that the fusion protein was correct. Both sera, the one against the GST-fusion protein and the one against the outer membrane preparation of *A. pleuropneumoniae*, were tested in a whole cell immuno dot blot mimicking the situation in the porcine lung. Neither of the sera was able to distinguish between whole cells of *A. pleuropneumoniae* wt and *A. pleuropneumoniae* Δ *frpB*. Therefore, the sera were non-applicable to immunohistochemistry (Fig. 11).

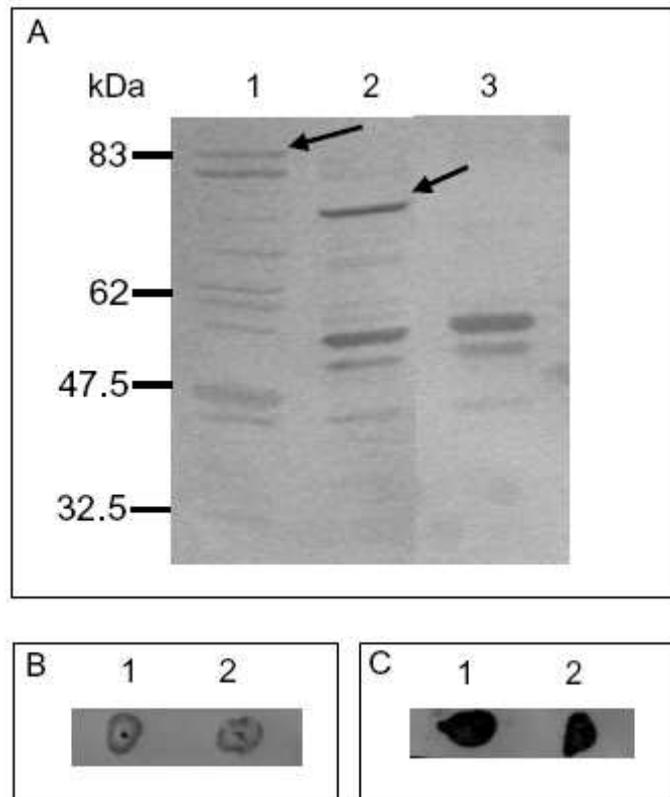


Fig. 11 Detection of FrpB in Western blot and immuno dot blot analyses. A) Western blot analysis with serum directed against the GST-FrpB fusion protein, 1) *E. coli* HB101 + pFrpB500, 2) *A. pleuropneumoniae* wt, 3) *A. pleuropneumoniae* Δ frpB; arrows indicate the GST-FrpB fusion protein (1) and the FrpB protein (2). The protein is absent in *A. pleuropneumoniae* Δ frpB (3). B) Whole cell immuno dot blot with serum directed against the GST-FrpB fusion protein, 1) *A. pleuropneumoniae* wt; 2) *A. pleuropneumoniae* Δ frpB; C) Whole cell immuno dot blot with serum against outer membranes of *A. pleuropneumoniae* wt, 1) *A. pleuropneumoniae* wt; 2) *A. pleuropneumoniae* Δ frpB.

4.3 The hypothetical protein Ape51 of *A. pleuropneumoniae*

The *ape51* gene of *A. pleuropneumoniae* has been shown to be located in the outer membrane and to be regulated by the global anaerobic regulator ArcA (BUETTNER 2008). In addition, it is located directly downstream of the *arcA* gene. Therefore, it was considered to be suitable target for immunohistochemistry aimed at the determination of ArcA-regulation in vivo. However, prior to use Ape51 as a target, its possible role in virulence and its function were to be determined using an isogenic deletion mutant.

4.3.1 Construction of an isogenic *A. pleuropneumoniae* Δ *ape51* strain

The DNA-regions upstream and downstream of the *ape51* gene were amplified from *A. pleuropneumoniae* chromosomal DNA with primer pairs oApe-1 and oApe-2 as well as oApe-3 and oApe-4, respectively. The resulting PCR products were digested with the restriction endonuclease BsmBI and ligated. The ligation product was again amplified via PCR with primers oApe1 and oApe4, followed by restriction of the PCR product with PspOMI and NotI. The PCR product was then ligated into pEMOC2 restricted with the PspOMI and NotI resulting in the transconjugation plasmid pAPE700 carrying a truncated *ape51* gene lacking 513 bps at positions 4 to 516 of the *ape51* ORF (Fig. 12).

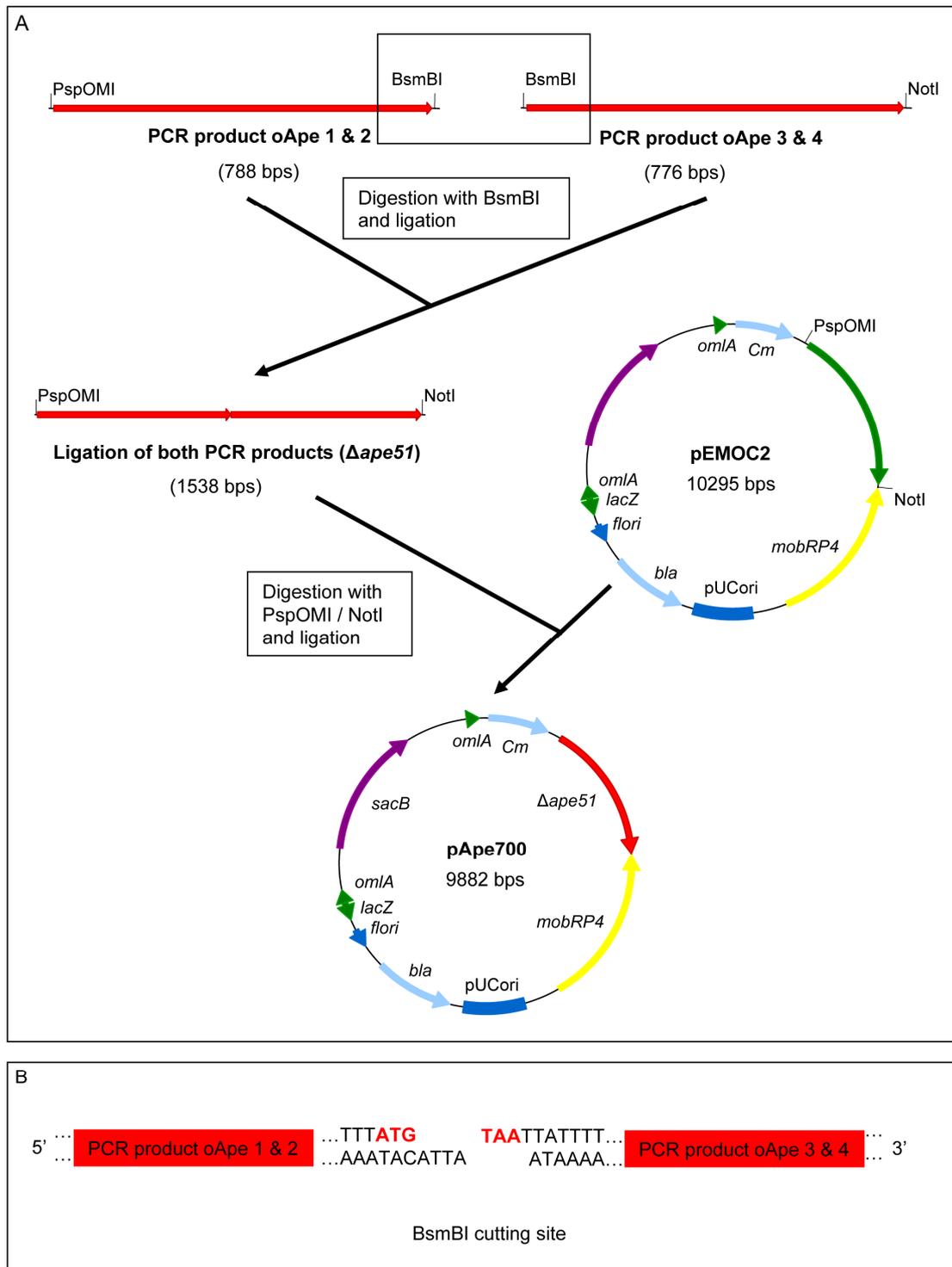


Fig. 12 Construction of pApe700. A) Arrows on the circular maps indicate the respective reading frames. *bla* beta-lactam antibiotic resistance determinant, *Cm* chloramphenicol resistance determinant, *sacB* counterselectable marker levansucrase of *B. subtilis*, insertion of Δ ape51 is highlighted in red; the boxed area is enlarged in (B); B) Schematic of BsmBI cutting site of PCR

products obtained with primers oApe 1 & 2 and oApe 3 & 4; red font shows the start and the stop codon of *ape51*.

The isogenic, in-frame *ape51* deletion mutant of *A. pleuropneumoniae* AP76 was constructed via allelic exchange using diaminopimelic acid auxotrophic *E. coli* β 2155 as donor for plasmid pAPE700. Confirmation of the plasmid's integration into the *A. pleuropneumoniae* chromosome was done using PCR on chloramphenicol resistant colonies. Clones with the right PCR profile were subjected to counterselection. Verification of mutants after counterselection was done by PCR with primers oApe-1 and oApe-4, Southern blot analysis and nucleotide sequencing. In addition, the absence of the Ape51 protein was confirmed by 2D PAGE.

The absence of gross genomic rearrangements was confirmed with PFGE (Fig. 13). The specificity of the deletion was confirmed by complementation of *A. pleuropneumoniae* Δ *ape51* with plasmid pAPE1300a and subsequent 2D-PAGE.

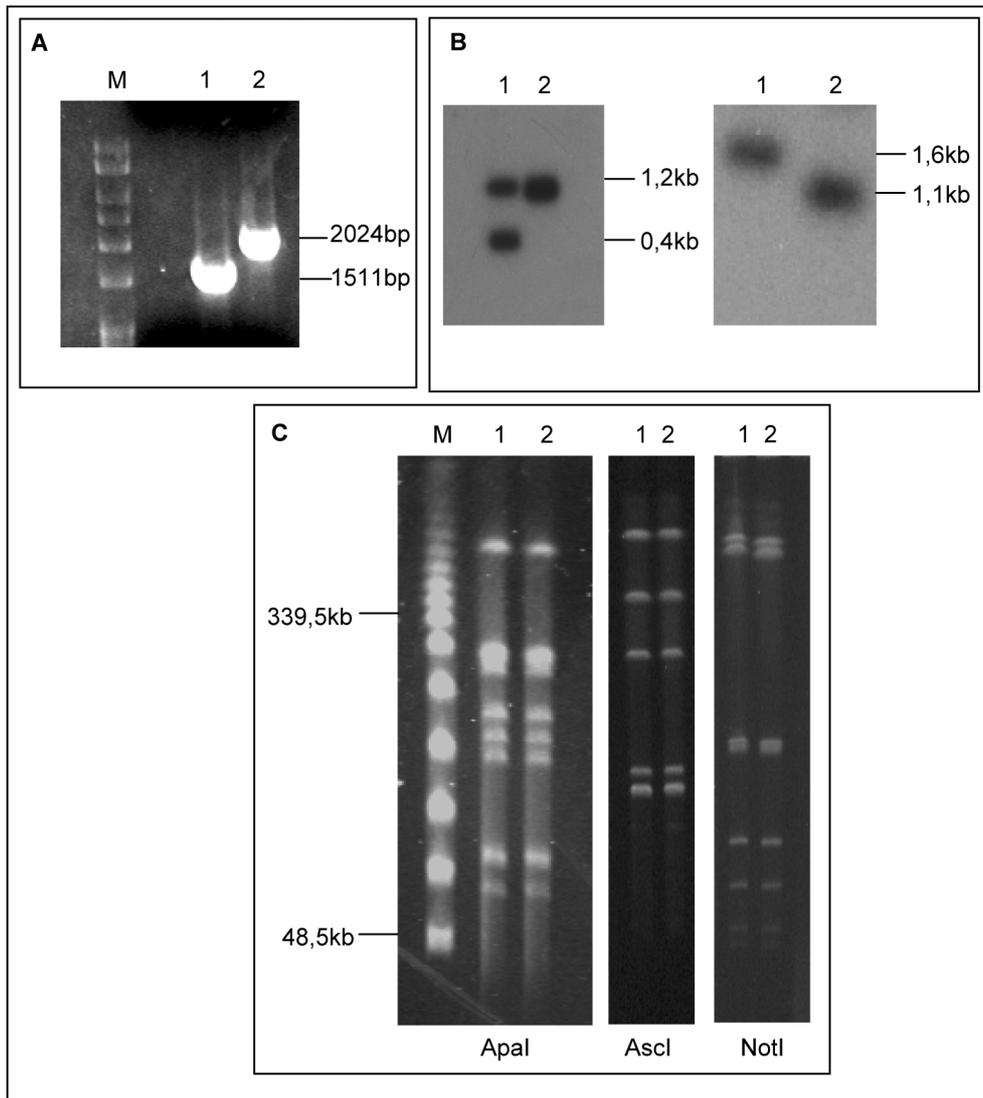


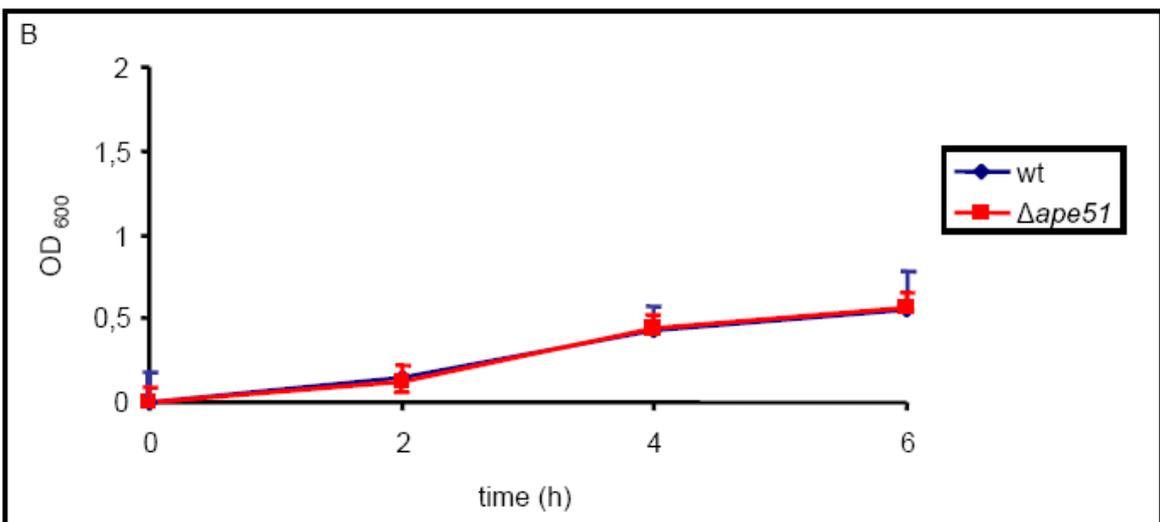
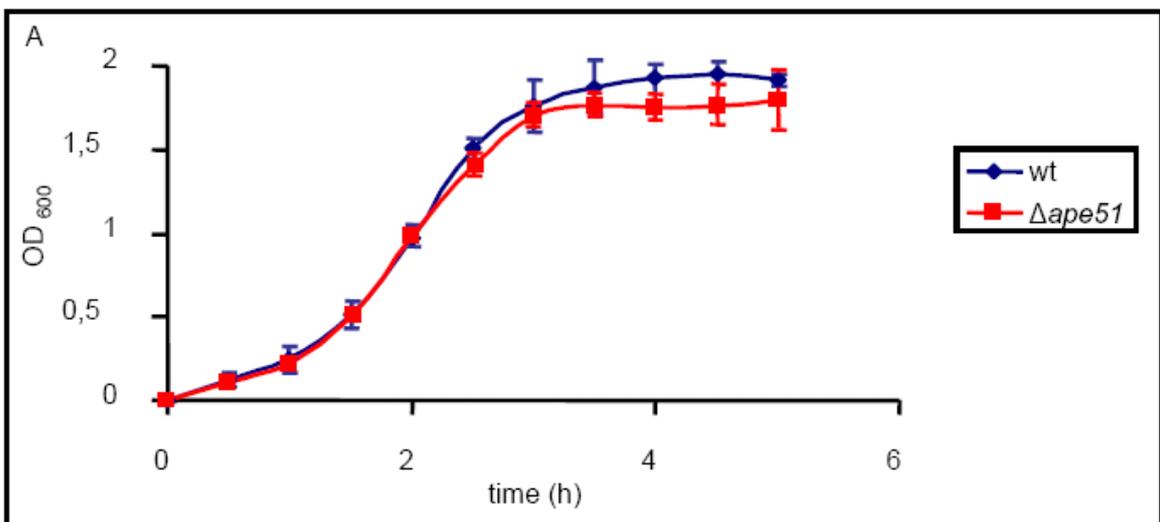
Fig. 13 Verification of Δ ape51 deletion by PCR, Southern blot analysis and PFGE. A) PCR analysis, products obtained with primers oApe-1 and oApe-4 from *A. pleuropneumoniae* genomic DNA, M) 100-5000 bp ladder; B) Southern blot analysis using radiolabeled PCR product of primers oApe-1 and oApe-4; restriction of genomic DNA with BsrGI / BsII (left) and BslI (right); C) PFGE to exclude genomic rearrangements M) phage lambda concatameres as size marker; for digestion restriction endonucleases Apal, Ascl and NotI were used as indicated. 1) *A. pleuropneumoniae* wt; 2) *A. pleuropneumoniae* Δ ape51.

4.3.2 Growth and survival characteristics of *A. pleuropneumoniae* Δ ape51

Growth curves of *A. pleuropneumoniae* Δ ape51 and *A. pleuropneumoniae* wt were compared under aerobic and anaerobic conditions. Under aerobic conditions both strains reached the exponential growth phase approximately 2 hours post inoculation

and stationary phase was reached 4 hours post inoculation. Likewise, growth behaviour of both strains did not differ under anaerobic conditions. As expected, growth of both strains under anaerobic conditions was slower than under aerobic conditions.

The survival kinetics of *A. pleuropneumoniae* Δ *ape51* were monitored in order to determine whether the *ape51* gene has an effect on bacterial aging. It was shown that both strains, *A. pleuropneumoniae* wt and *A. pleuropneumoniae* Δ *ape51* were culturable until up to 30 hours post inoculation in PPLO medium under aerobic as well as anaerobic conditions with only minor differences in survival between *A. pleuropneumoniae* wt and *A. pleuropneumoniae* Δ *ape51* (Fig. 14).



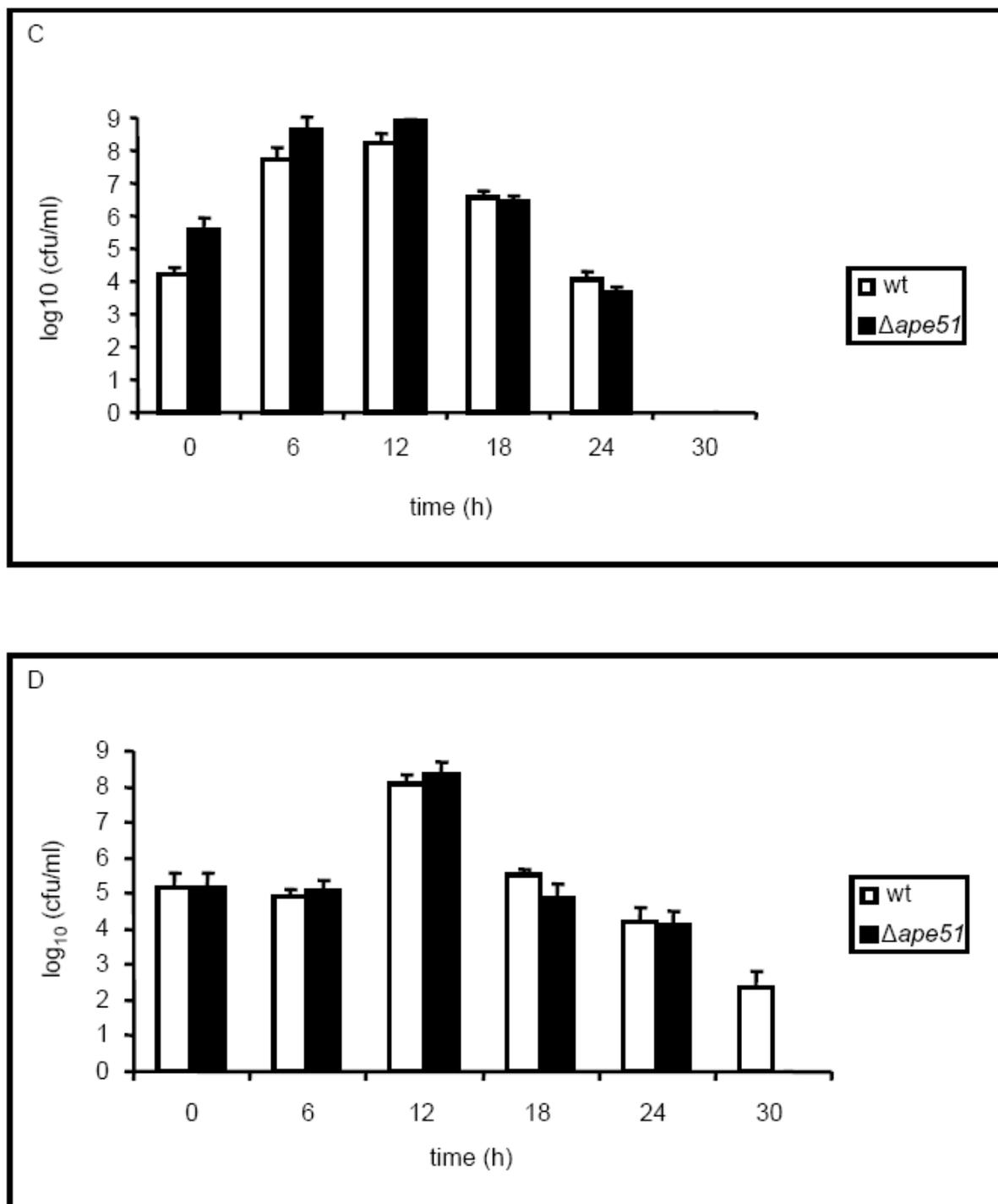


Fig. 14 Analysis of growth and survival of *A. pleuropneumoniae* wt and Δ ape51. A) Aerobic growth; B) anaerobic growth; C) aerobic survival; D) anaerobic survival. Differences in growth and survival characteristics are statistically insignificant. Bars represent the arithmetic means and hinges the standard deviation as determined from three independent measurements.

4.3.3 Transcriptional analysis of the *A. pleuropneumoniae* *ape51* gene

It was hypothesised that the *ape51* gene might have an effect on the anaerobic metabolism of *A. pleuropneumoniae* because it is situated directly downstream of the *arcA* gene. Therefore, *A. pleuropneumoniae* wt RNA was analysed in order to determine whether the *ape51* gene and the preceding *arcA* gene are co-transcribed. Primer pairs were positioned within the *arcA* gene, the intergenic region between the two genes and within the *ape51* gene itself. In a reverse transcriptase PCR reaction it could be shown that both genes are encoded on the same polycistronic RNA (Fig. 15).

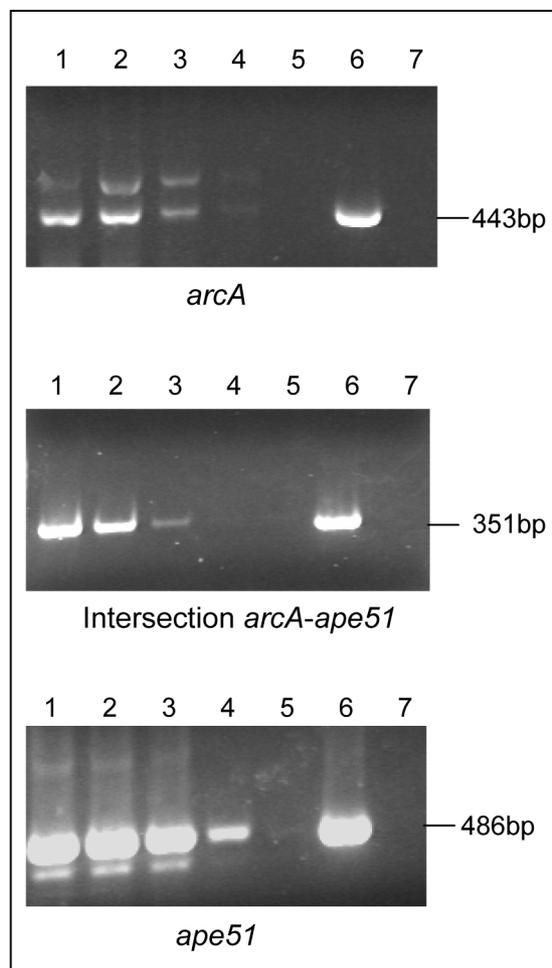


Fig. 15 The *arcA* and the *ape51* genes of *A. pleuropneumoniae* are transcribed on one polycistronic RNA. 1,2,3,4 cDNA of the respective genes and the intergenic region between both; 1) undiluted; 2) diluted 1:10; 3) diluted 1:100; 4) diluted 1:1000; 5) control without reverse transcriptase; 6) chromosomal DNA *A. pleuropneumoniae* wt (positive control); 7) water (negative control).

4.3.4 Expression of the *A. pleuropneumoniae* Ape51 protein under aerobic and anaerobic conditions

Protein expression of *A. pleuropneumoniae* wt under aerobic and anaerobic growth conditions was compared using 2D PAGE with respect to differences in the expression of the Ape51 protein. It could be shown that Ape51 is expressed solely under anaerobic conditions (Fig. 16), thereby supporting previous results showing that the protein is expressed only in the presence of ArcA and showing, that the protein might be a suitable target to exemplarily investigate the expression of ArcA regulated proteins in vivo.

Prior to using Ape51 as a marker protein, its potential function as well as its role in virulence were to be determined.

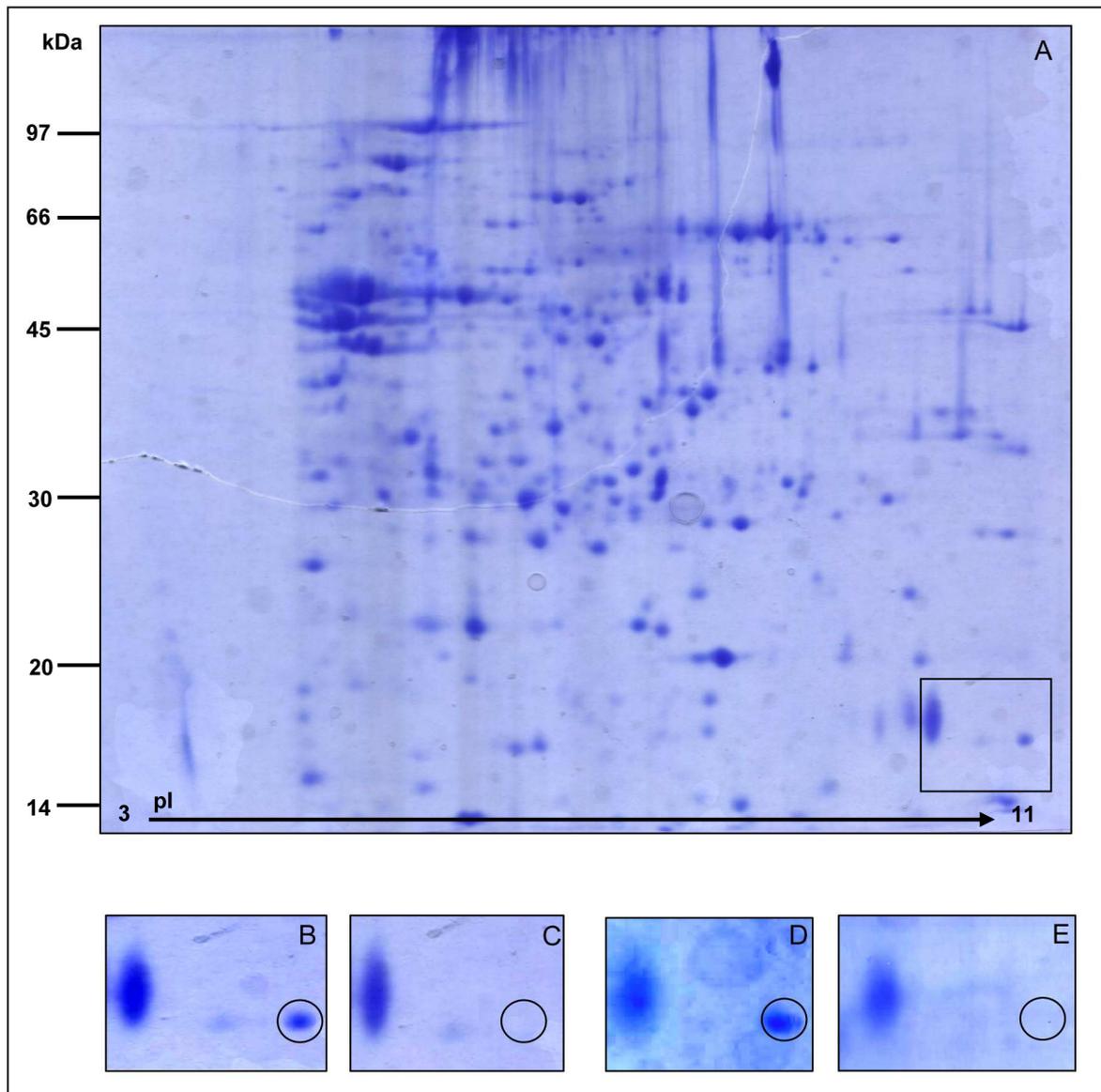


Fig. 16 2D-PAGE; Expression of the Ape51 protein under aerobic and anaerobic growth conditions. A) Gel loaded with protein preparation of *A. pleuropneumoniae* culture supernatant, first dimension focussing was done on a linear Immobiline®DryStrip, pH 3 to 11. The boxed area is enlarged in B to E. B) *A. pleuropneumoniae* wt, anaerobic growth conditions, C) *A. pleuropneumoniae* Δ *ape51*, anaerobic growth conditions, absence of the protein in the deletion mutant D) *A. pleuropneumoniae* Δ *ape51* complemented with pApe1300a, anaerobic growth conditions, E) *A. pleuropneumoniae* wt, aerobic growth conditions. Black circles indicate the (potential) position of the Ape51 protein. Numbers to the left indicate the size in kilo Dalton (kDa), the line on the bottom shows the isoelectric point's gradient.

4.3.5 Adherence of Δ *ape51* to living-related lung tissue slices

A BLAST search with the amino acid sequence of the Ape51 protein revealed a homology to an OmpA-like protein in *Haemophilus (H.) ducreyi* (KOMATSUZAWA et al. 2002) which is known to be involved in adherence to host epithelial surfaces during the first phase of infection. In order to investigate a possible role of the Ape51 protein in adherence an assay with living lung tissue slices containing intact bronchial surface structures was performed.

Tissue slices of the lung were cut such, that bronchioli were preferably located in the centre of the slices. Cilial movement, or stop of cilial movement, were defined as parameters for monitoring adherence and toxic effect of *A. pleuropneumoniae*, as the bronchial epithelial surface is the location for adherence during natural infection in the pig. In the experimental set-up with three different groups (group 1 infected with *A. pleuropneumoniae* wt, group 2 infected with *A. pleuropneumoniae* Δ *frpB*, group 3 infected with *A. pleuropneumoniae* Δ *ape51*) were compared to uninfected tissue slices for 24 hours.

The duration of cilial movement was indistinguishable in all groups. Stop of cilial movement in all tissue slices was observed after 24 hours while it continued in the uninfected control group (Fig. 17). These results indicate that the chosen cell culture system is not suitable as a model for monitoring differences in adherence of *A. pleuropneumoniae* to its target organ.

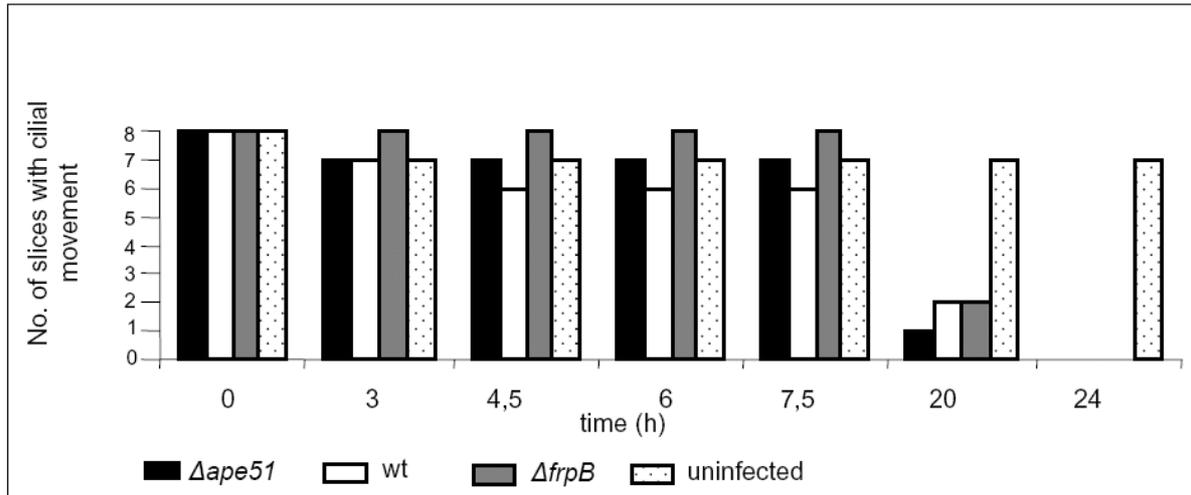


Fig. 17 Adherence of $\Delta ape51$ to living-related lung tissue slices. Comparison of cilia movement of *A. pleuropneumoniae* wt, *A. pleuropneumoniae* $\Delta ape51$ and *A. pleuropneumoniae* $\Delta frpB$ with non-infected tissue slices. The infected slices show insignificant differences in cilia movement when the 3 groups are compared with each other. The uninfected group represents the negative control.

4.3.6 Virulence studies

Since the tissue slice model was shown to be unsuitable for the investigation of *A. pleuropneumoniae* virulence an aerosol infection experiment with *A. pleuropneumoniae* $\Delta ape51$ was performed. One group of pigs (16 animals) was infected with *A. pleuropneumoniae* wt (1.69×10^5 aerosolised cfu for 4 pigs), a second group (16 animals) with *A. pleuropneumoniae* $\Delta ape51$ (4.2×10^4 aerosolised cfu for 4 pigs). Twelve pigs of the wild type and eleven pigs of the $\Delta ape51$ group were sacrificed on day 4 post infection to monitor the effects of infection during the acute phase of disease, four randomly assigned pigs of each group were sacrificed after 21 days during the chronic phase of disease. One *A. pleuropneumoniae* $\Delta ape51$ -infected pig was euthanised on day one after infection due to severe clinical symptoms. A rise of body temperature above 40°C after infection with the wild type strain was observed in 8 out of 16 pigs compared to only 2 out of 16 pigs infected with *A. pleuropneumoniae* $\Delta ape51$. No differences in clinical symptoms such as coughing, dyspnoea and vomiting were observed (Fig. 18).

Post mortem evaluation of the lungs revealed no alteration of lung tissue in 10 of 16 pigs in the *A. pleuropneumoniae* wt-infected group, in the *A. pleuropneumoniae*

$\Delta ape51$ -infected group 13 animals showed no lung alterations. Both the difference in the number of pigs with increased temperature after infection and the number of pigs with lung lesions implied a slight attenuation of *A. pleuropneumoniae* $\Delta ape51$; however, the differences were not statistically significant.

Both strains, *A. pleuropneumoniae* wt and *A. pleuropneumoniae* $\Delta ape51$ were consistently reisolated. This showed that all animals had in fact been infected but supported the finding that attenuation of *A. pleuropneumoniae* $\Delta ape51$ was not significant (Fig. 18, Table 7).

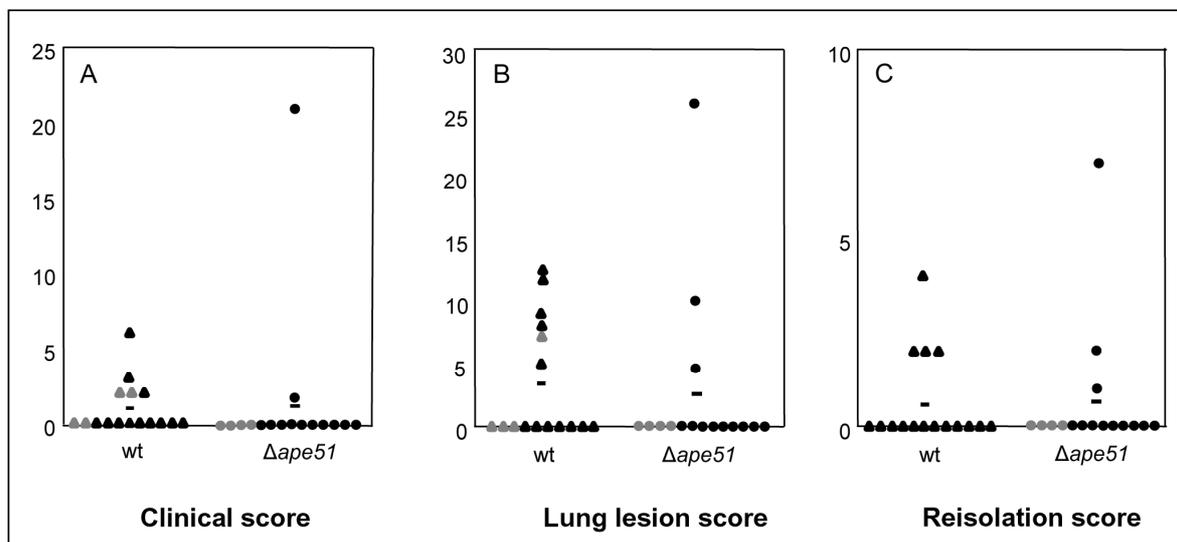


Fig. 18 Virulence of *A. pleuropneumoniae* wt and *A. pleuropneumoniae* $\Delta ape51$ compared in an aerosol infection model. Clinical score (A), lung lesion score (B) and reisolation score (C) of pigs infected with *A. pleuropneumoniae* wt (▲ [sacrificed on day 7 p.i.]; △ [sacrificed on day 21 p.i.]) or $\Delta ape51$ (● [sacrificed on day 7 p. i.]; ○ [sacrificed on day 21 p.i.]). Each triangle or circle represents one animal. The horizontal line represents the arithmetic mean. Probabilities as obtained using the Wilcoxon Signed-Rank Test did not show statistically significant differences between the groups. The animals sacrificed at different time points post infection were considered as a single group since both lung lesion and reisolation scores do not increase after day 7 of an *A. pleuropneumoniae* infection. Serum samples were analysed for presence of antibodies against *A. pleuropneumoniae* one week prior to infection and no *A. pleuropneumoniae* specific antibodies could be detected.

Table 7 Virulence of *A. pleuropneumoniae* wt and Δ *ape51* after aerosol challenge.

Challenge strain	No. of animals	Necropsy time p.i.	No. of animals with lung lesions	No. of animals and location of reisolation of <i>A. pleuropneumoniae</i> at post mortem analysis			
				tonsil	lymph node	lung	
						pneumonic	intact
<i>A. pleuropneumoniae</i> wt	12	day 7	5/12	7/12	5/12	3/12	4/12
	4	day 21	1/4	1/4	1/4	1/4	0/4
<i>A. pleuropneumoniae</i> Δ <i>ape51</i>	11 ^a	day 7	2/11	6/11	3/11	1/11	3/11
	4	day 21	0/4	0/4	0/4	0/4	0/4

^a One pig was euthanised on day 1 p. i. because of severe clinical symptoms. This pig had a lung lesion score of 25.8. The challenge strain was reisolated in lymph node, pneumonic and intact lung.

4.3.7 Detection of the Ape51 protein with polyclonal antiserum

As described for the FrpB protein it was a goal of the study to investigate expression of HlyX- and ArcA-regulated proteins in vivo. Although no decisive function or role in pathogenicity could be shown for the Ape51 protein it was considered to be suitable for the expression study as it was clearly regulated under anaerobic conditions. Furthermore, previous work using HlyX and ArcA mutants had shown that Ape51 is not regulated by HlyX so that differences in expression are likely to be predominantly ArcA-dependent.

Therefore, part of the *ape51* gene was cloned into an expression vector facilitating inducible expression of a GST-Ape51 fusion protein with a total size of 39 kDa. Polyclonal antibodies were raised against the GST-Ape51 fusion protein in rabbits for detection of linearised epitopes of the denatured protein. Pooled sera of pigs convalescent from Porcine Pleuropneumonia were used in order to detect conformational epitopes present in the native protein localised in the outer membrane. These antibodies were required to semiquantitatively determine the protein in Western blot analysis as well as in formaldehyde-fixed lung tissue slices of infected pigs. Purification of antibodies was done via adsorption against denatured whole cell lysates of *E. coli* HB101 carrying pGEX5x3 and against denatured whole

cell lysates of *A. pleuropneumoniae* Δ *ape51* in case of the GST-fusion protein, the convalescent sera were adsorbed against formaldehyde-fixed bacteria. Adsorptions were done to reduce unspecific background signals. Both sera were further purified with a protein G column.

The GST-Ape51 fusion protein as well as the native Ape51 protein were recognised by the serum directed against the GST-Ape51 fusion protein in Western blot analysis. To test whether the two sera would likewise specifically recognize the non-denatured protein (as required for immunohistochemistry), a whole cell immuno dot blot was performed. It could be shown that both are not able to distinguish between *A. pleuropneumoniae* wt and the Δ *ape51* mutant making the serum unfeasible for immunohistochemistry (Fig. 19).

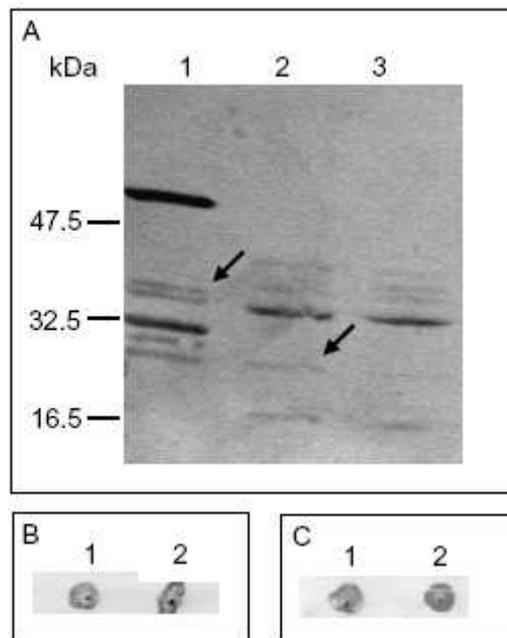


Fig. 19 Detection of Ape51 in western blot and immuno dot blot analyses. A) Western blot analysis with serum directed against the GST-Ape51 fusion protein, 1: *E. coli* HB101 + pApe500, 2) *A. pleuropneumoniae* wt, 3) *A. pleuropneumoniae* Δ *ape51*; arrows indicate the GST-Ape51 fusion protein (1) and the Ape51 protein (2). The protein is absent in *A. pleuropneumoniae* Δ *ape51* (3). B) Whole cell immuno dot blot with serum directed against the GST-Ape51 fusion protein, 1) *A. pleuropneumoniae* wt; 2) *A. pleuropneumoniae* Δ *ape51*; C) Whole cell immuno dot blot with convalescent serum, 1) *A. pleuropneumoniae* wt; 2) *A. pleuropneumoniae* Δ *ape51*.

5 Discussion

A. pleuropneumoniae, the causative agent of Porcine Pleuropneumonia, is a highly contagious gram-negative bacterium responsible for high economic losses in the pig industry. In order to better understand the underlying mechanisms of the disease several virulence factors have been investigated. Here, iron uptake and its regulation have been shown to be crucial for survival of the bacterium in the host (BALTES et al. 2002; JACOBSEN et al. 2005a), and - although the natural niche of *A. pleuropneumoniae* is the porcine lung - adaption to anaerobic metabolism is also clearly virulence-associated (BALTES et al. 2003a; BALTES et al. 2005; BUETTNER et al. 2008b; BUETTNER et al. 2008a; BUETTNER 2008).

The aim of this study was to further characterise the iron-dependent and anaerobic metabolism under in vivo and in vitro conditions. An *A. pleuropneumoniae* GFP marker strain was constructed in order to track the bacterium in the host when iron is scarce. Furthermore, the role of the FrpB protein in iron uptake and its influence in lung tissue damage was assessed. With respect to anaerobic metabolism expression of the Ape51 protein and its potential function were investigated.

5.1 In vivo tracing of the *A. pleuropneumoniae* GFP marker strain

Before tracing of bacteria from infected animals by FACS had been accomplished (BUMANN 2001) different approaches towards monitoring host-pathogen interactions with FP marker strains had been achieved. Thus, fluorescent proteins have been used to directly label *Mycobacterium marinum* and to visualise the bacterium in sections of infected liver and spleen. It was shown that the plasmid-based expression of GFP did not alter the pathohistological findings usually seen in *Mycobacterium* infection, and fluorescence was detected consistently from all bacteria (VALDIVIA et al. 2006). A similar result was found in *Streptococcus suis* where pigs were infected with a GFP marker strain which could be detected in tissue slices of the tonsils up to 168 h after infection by fluorescence microscopy (LUN et al. 2003). Furthermore the reliable recovery of bacteria from liquid cultures is facilitated by FACS. The recovery rate for GFP labelled *S. Typhimurium* in liquid culture was 85 % in a plasmid-based

approach where the *gfp* gene is present in multiple copies (VALDIVIA et al. 1996). In chromosomal fusions where the *gfp* gene is present only once in the cell the average recovery rate declines because of the lower fluorescence intensity compared to a plasmid-based approach. In an *E. coli* strain with a chromosomal GFP fusion the average recovery rate was 55 % (FERRARI et al. 2004). When comparing a plasmid-based approach with a chromosomal fusion, the advantage of plasmid encoded *gfp* certainly is the brighter fluorescence but it has been shown that plasmids are potentially unstable, and high expression can lead to plasmid loss while chromosomal fusions are stably maintained (HAUTEFORT et al. 2003; HOPKINS et al. 2000). In other studies it was possible to detect cells infected with GFP marker strains by FACS. Thus, macrophages infected with fluorescent *Yersinia pseudotuberculosis* and *Listeria monocytogenes* were sorted and could be shown to provide a useful tool to differentiate infected from uninfected macrophages for monitoring adhesion and invasion in a cell culture model and to sort for virulence induced genes (VALDIVIA et al. 1996; WILSON et al. 2001). Furthermore, HAUTEFORT et al. (2003) described that chromosomal single-copy *gfp* fusions allow the FACS detection of in vitro induced virulence genes in *S. Typhimurium*. As it was possible to detect *gfp* labelled bacteria by FACS and monitor the expression of their virulence genes in cell culture models in vitro, it was desirable to analyse infected host tissue directly by FACS sorting, thereby transferring the method into an in vivo setting. The principle feasibility of such an approach was demonstrated by BUMANN (2001) who infected mice intragastrically with *Salmonella* Typhimurium carrying a medium-copy-number plasmid with a GFP fusion protein which facilitated detection of the bacteria in the Peyer's Patches of the ileum. A transfer of this method to an *A. pleuropneumoniae* infection model was conducted in this study. To circumvent the disadvantages of a plasmid-based construct a chromosomal GFP fusion was used.

Iron limitation is known to be a factor influencing *A. pleuropneumoniae* metabolism in vitro. However, little is known about iron-induced changes in *A. pleuropneumoniae* metabolism in vivo. Therefore, in this study an *A. pleuropneumoniae* GFP marker strain has been constructed by introducing a *gfp* gene into the *tbp* operon encoding the transferrin-binding proteins in order to monitor the effects of iron limitation in the

host. The marker strain exhibited characteristics indistinguishable from the parent strain with respect to acquisition of iron from porcine transferrin in an in vitro assay. Furthermore, fluorescence microscopy showed that – upon induction of iron restriction – all bacteria showed comparable fluorescence intensity. These were promising results because i) they show that the artificial operon constructed is fully functional, and ii) uniform fluorescence is required for reliable FACS sorting. Plasmid-based expression of GFP has been shown to cause highly heterogeneous expression in individual bacteria which impedes sorting by FACS (THONE et al. 2007). An animal experiment with pigs was accomplished because host induced changes of *A. pleuropneumoniae* iron metabolism can best be monitored in the natural host. The GFP marker strain was used in an aerosol infection experiment where it induced severe clinical symptoms in pigs thereby showing that it was still fully virulent. This confirmed the in vitro results on the function of the artificial operon as prevention of iron uptake from transferrin would have rendered the bacterium avirulent (BALTES et al. 2002). The bacteria which were reisolated from the porcine lung were positively tested for GFP fluorescence proving that the chromosomal integration of GFP is stably maintained through several generations in vivo. Together, these results show that foreign DNA can be inserted into the *A. pleuropneumoniae* *tbp* operon and that the encoded proteins are expressed without altering the *A. pleuropneumoniae* characteristics with respect to iron acquisition and virulence.

The aim of the study was to quantify the proportion of bacteria expressing Tbp proteins by comparing the number of fluorescent bacteria determined by FACS analysis with the total number of viable bacteria determined by culture. As no experience was available on the sorting of bacteria from lung tissue, and since the feasibility of the approach largely depends on the in vivo expression signal of the bacteria which cannot be determined in vitro, an initial animal experiment was performed. It was shown that bacterial expression of GFP was low in comparison to background autofluorescence (high signal to noise ratio). Thus, the lung particles emitted an intense yellow-green autofluorescence which prevented gating for the GFP marker strain and, therefore, FACS-counting of GFP-positive bacteria could not be performed successfully. Enhancement of fluorescence intensity would have been essential to overcome the high signal to noise ratio. This might have been achieved

through a plasmid-based GFP marker strain but the application of such a construct would have led to the above mentioned disadvantages. The use of differently coloured FPs was not feasible either as they overlap with the spectral range of autofluorescence (Bumann, D., personal communication). Since no suitable technology was available to sufficiently improve the signal-to-noise ratio to facilitate FACS sorting this approach was not pursued any further. The initial approach, in retrospective, is supported by the fact that THONE et al. (2007) recently showed that FACS-sorting of bacteria carrying a single copy of the *gfp* gene on the chromosome is possible. However, more than 100 different constructs had to be investigated which justifies the decision to discontinue this approach and investigate the feasibility of different methodologies.

The aim of infecting pigs with an *A. pleuropneumoniae* GFP marker strain was to monitor the proportion of bacteria expressing Tbp proteins under iron restricted conditions in vivo as it presents an indicator for the activity of the global regulator Fur in individual bacteria. Since it was not possible to detect the bacteria in lung homogenates by FACS analyses immunohistochemistry was approached as an alternative.

In immunohistochemistry the visualisation of green-fluorescent *A. pleuropneumoniae* in the lung can be combined with the immunostaining of all *A. pleuropneumoniae* bacteria present in the slice using an antibody directed towards a constitutively expressed and surface-exposed protein. Recently, the technology has been improved by the development of photoactivatable GFPs. As these GFPs are induced by a different wave length the signal-to noise ratio is up to 100-fold lower than for wild type GFP when first irradiated with 413 nm light and then excited with light of a 488 nm wavelength (PATTERSON and LIPPINCOTT-SCHWARTZ 2002). The increased contrast between the surrounding tissue and bacteria might facilitate successful detection of the marker strain in the lung. However, the expression of these GFPs in bacteria has not been investigated to date.

Since the microscopic detection of GFP-labelled bacteria was not successful, a commercial monoclonal antibody was used to detect denatured GFP in a Western

blot analysis to see whether it is possible to detect the protein in lysed cells. However, detection was unsuccessful probably because the antibody was optimised for use in eukaryotic cells (personal communication, Invitrogen™). This can only be explained by potential differences in GFP glycosylation; however, such modifications of GFP have not been described to date. Thus, secondary detection of GFP would have facilitated a quantification of bacteria without being able to localise them histologically. The use of a commercially available antibody would have compensated for the time and effort of raising polyclonal antibodies in rabbits. As, however, the secondary antibody did not bind, it was decided to raise antibodies against an appropriately regulated outer membrane protein as this would facilitate detection of intact bacterial cells and histological evaluation.

As the proportion of Tbp expressing bacteria in vivo (serving as a measure of activity of the regulator Fur) could not be determined using a GFP-marker strain the method was not tried for the investigation of in vivo regulation by the regulators HlyX and ArcA. Instead it was decided to use conventional immunohistochemistry using specific antibodies which target a highly regulated outer membrane protein of the respective regulon as this methodology had been used successfully for initial investigations of Fur activity in vivo (HENNIG et al. 1999). The two candidate proteins used were the FrpB protein which was shown to be 8.8 fold regulated by the FNR-homologue HlyX (BUETTNER 2008) and the Ape51-protein which was regulated 1.8 fold by ArcA and located directly downstream of the *arcA* gene on the chromosome (BUETTNER 2008). Before generating antibodies against these proteins and using them in immunohistology, the functional significance of both proteins was to be determined.

5.2 The FrpB protein of *A. pleuropneumoniae*

The iron-regulated outer membrane protein (FrpB) is a 73 kDa outer membrane protein of *A. pleuropneumoniae* that was identified to be involved in virulence as an *frpB* deletion mutant was shown to be avirulent in an animal infection experiment (BENDALLAH 2008). This result was underlined by the results in this work that virtually no alterations of lung tissue could be detected in histopathological analyses.

Investigation of the function and regulatory mechanisms revealed that the FrpB protein is essential for the utilisation of transferrin-bound iron by *A. pleuropneumoniae* as the *frpB* deletion mutant was not able to utilise iron-saturated porcine transferrin as an iron source. This finding may be highly significant since the exact means of transport of transferrin-bound iron through the membranes are not known to date (PERKINS-BALDING et al. 2004). Thus, *A. pleuropneumoniae* and other bacteria such as *Neisseria (N.) meningitidis* and *Haemophilus influenzae* bind transferrin via a complex of their large and small transferrin binding proteins (GONZALEZ et al. 1990; LEGRAIN et al. 1993; SCHRYVERS 1989). Both proteins are located in the outer membrane. The small transferrin-binding protein is a lipoprotein of 60 to 70 kDa (PERKINS-BALDING et al. 2004), and the large transferrin-binding protein is an integral membrane protein of approximately 100 kDa (PERKINS-BALDING et al. 2004). However, the way how iron is removed from transferrin and transported across the outer membrane into the periplasm remains unclear (PERKINS-BALDING et al. 2004). The FrpB homologue in *Neisseria (N.) gonorrhoeae* is a 76 kDa outer membrane protein with a sequence identity of only 33 % to *A. pleuropneumoniae* FrpB (BEUCHER and SPARLING 1995). Deletion of *frpB* in *N. gonorrhoeae* revealed that neisserial FrpB – other than in *A. pleuropneumoniae* - is not required for acquisition of transferrin-bound iron therefore differing in its function from *A. pleuropneumoniae* FrpB (BEUCHER and SPARLING 1995). Further, amino acid sequence analysis revealed a 31 % identity of FrpB to IrpA of *Moraxella (M.) bovis*, a 79 kDa outer membrane protein which has been shown to be involved in uptake of transferrin-bound iron in this organism. Thus, a *M. bovis irpA* mutant was shown to be impaired but not completely disabled in its ability use transferrin (KAKUDA et al. 2003). It is suggested that FrpB may be a transport protein for a low-affinity siderophore (CARSON et al. 1999) required for uptake of iron released from transferrin. Such low affinity siderophores have been described for *A. pleuropneumoniae* (DIARRA et al. 1996) and the difference in phenotype to the respective mutants in *N. gonorrhoeae* and *M. bovis* may be due to back-up transport systems in these organisms.

Furthermore the regulation of the FrpB protein was analysed. It is classified in the data base as Iron-Regulated Protein B but was identified in *A. pleuropneumoniae* as

strongly upregulated (2.6-fold on transcriptional level and 8.8-fold on the protein level) by the anaerobic regulator HlyX (BUETTNER 2008). Therefore, it was investigated whether i) deletion of the *frpB* gene would hamper growth under anaerobic conditions and ii) whether expression of FrpB protein was regulated not only by HlyX but also by the Ferric uptake regulator protein Fur. The results showed that *A. pleuropneumoniae* wt and the *frpB* mutant – like under aerobic conditions (BENDALLAH 2008) - did not reveal any significant differences with respect to growth and survival under anaerobic conditions in vitro. It was concluded that the attenuation of the *frpB* mutant is not due to growth deficiencies under anaerobic conditions in the host but dependent on the inability to use transferrin as iron source. An investigation of the iron regulation - implied by the designation of the protein - was performed in silico and by using an *A. pleuropneumoniae fur* mutant. The in silico analysis revealed that no Fur box was present upstream of the start codon as it has been described for *N. gonorrhoeae* (BEUCHER and SPARLING 1995) and *M. bovis* (KAKUDA et al. 2003). Further, expression experiments using the *fur* mutant showed no significant regulation of the FrpB protein. These results clearly underline regulatory differences between the homologous proteins which may be due to coordinated regulation for FrpB (or homologue) by more than one regulator in other organisms (DELANY et al. 2006). Together, these results show that the designation of FrpB in *A. pleuropneumoniae* – despite its clear involvement in the utilisation of transferrin-bound iron - is misleading as no evidence for a direct regulation by Fur could be detected.

After having determined the function of the FrpB protein, the antibodies raised against both, outer membranes of anaerobically grown *A. pleuropneumoniae* and against a recombinant fusion protein, were tested with respect to their applicability in immunohistology. It was found, that neither of the two sera was specific and, unfortunately, specificity could not be obtained even after several different adsorption steps with different cross-reacting protein-preparations. It could be shown that the denatured linearised FrpB epitope was detected in a Western blot analysis with the serum raised against the GST-FrpB fusion protein. The same serum failed to specifically detect the FrpB protein in an immuno dot blot with intact bacteria. This indicated that the serum recognises linearised FrpB epitopes but fails to specifically

recognise conformational epitopes on intact bacteria. In this case, the lack of binding would be due to conformational differences between the native protein and the GST fusion protein. In summary detection of intact bacteria is facilitated but remains unspecific. In *N. meningitidis* it was shown that sera of mice immunised with whole bacteria could detect the conformational TbpA protein in a dot blot assay but failed to recognise the denaturated TbpA in Western blots (ALA'ALDEEN et al. 1994). A similar problem was addressed by WILKE et al. (1997). Pigs were immunised with either recombinant *E. coli* outer membrane preparations expressing the TbpB protein of *A. pleuropneumoniae* or soluble protein aggregates with the same protein. The sera of animals immunised with outer membrane preparations specifically detected those and not the protein aggregates in an ELISA and vice versa. Cross reactivity of antibodies between those two preparations could not be found. These results support the findings of this work and underline the problems occurring when investigating bacterial protein expression in vivo.

5.3 The hypothetical protein Ape51 of *A. pleuropneumoniae*

The *ape51* gene is located directly downstream of the *arcA* gene encoding an important anaerobic regulator. Most important for this study the Ape51 protein was found to be immunogenic with sera of convalescent pigs and to be expressed under anaerobic conditions whereas, in an *arcA* mutant, it was expressed constitutively (BUETTNER 2008). This strongly implied an ArcA-dependent regulation and, therefore, the Ape51 protein was a good candidate to investigate the activity of the regulator ArcA in vivo by immunohistology. Again, prior to raising specific sera, the role of the protein in vitro and in vivo was to be investigated. Upon construction of an isogenic *A. pleuropneumoniae* *ape51* deletion mutant and confirming its genotype (PCR, Southern Blot, PFGE) and phenotype (2D PAGE), growth and survival experiments under both aerobic and anaerobic conditions did not reveal any differences between wt and mutant in vitro. In addition, a pig infection experiment only provided an indication (reduced temperature increase after infection) that the *ape51* gene might be involved in virulence. A major cause for this unsatisfactory result was the unusually low number of diseased animals in the control group. As no

methodological reason for this result could be identified, it was hypothesised that the lack of clinical symptoms and lung lesions might be due to a higher natural resistance of the pigs; such a difference in resistance in different families of the breeding line of German Landrace used for the experiments had been observed previously (Hoeltig 2008, thesis in preparation).

As the Ape51 protein was found to be homologous to an OmpA-like putative adhesin in *H. ducreyi* (KLESNEY-TAIT et al. 1997) its possible role in adherence was investigated but no function could be observed. These results again show that designations given based on homologies frequently can be misleading with respect to function. However, the Ape51 protein was detected by convalescent serum (thereby proven to be immunogenic), and serum which was raised against recombinant GST fusion proteins. Unfortunately, these sera were, like the sera against the FrpB protein, not sufficiently specific. Although the serum raised against the Ape51-GST fusion protein recognised the denatured epitope in Western blot analysis the conformational epitope in an immuno dot blot could not be confirmed. Even several rounds of adsorption against the *A. pleuropneumoniae ape51* mutant did not lead to a clear differentiation between *A. pleuropneumoniae* wt and the *ape51* mutant, both grown under anaerobic conditions, in an immuno dot blot analysis. This again documents the difficulties in the antibody-mediated detection of bacterial membrane proteins in vivo.

In this project a GFP marker strain was constructed and shown to be stable, fully functional, and virulent. However, the level of GFP expression was not sufficiently high to facilitate FACS sorting of GFP-expressing bacteria from lung tissue. Therefore, it was attempted to investigate the in vivo-activity of the anaerobic regulators HlyX and ArcA by immunohistochemistry using the HlyX-regulated FrpB and the ArcA-regulated Ape51 proteins as targets. It was shown that the FrpB protein – despite its designation – is not regulated by Fur and that it is essential for the utilisation transferrin-bound iron. Unfortunately, the in vivo detection and quantification of FrpB failed as specific sera could not be obtained. For Ape51 the regulating influence of ArcA could be confirmed, as immunodetection only succeeded upon growth under anaerobic conditions. An adherence assay did not give insights

into Ape51 function, and an animal experiment only showed a slight attenuation of the *ape51* deletion mutant. The in vivo detection and quantification of Ape51 failed as the serum raised did not show sufficient specificity.

6 Summary

Gene regulation in *Actinobacillus pleuropneumoniae* -
Different approaches to determine the response to iron limitation
and anaerobiosis in vivo

Karla Dreckmann

The causative agent of Porcine Pleuropneumonia, *Actinobacillus* (*A.*) *pleuropneumoniae*, is a gram-negative bacterium responsible for high economical losses in the pig industry. To better understand the mechanisms of disease the iron- and anaerobiosis-dependent gene regulation was to be further characterised under in vivo and in vitro conditions.

In order to monitor the effects of iron limitation an *A. pleuropneumoniae* GFP marker strain was constructed, carrying the *gfp* gene as part of the *tbp* operon on the chromosome. Uniform GFP expression was induced by iron restriction in vitro, the insertion did not alter the strain's ability to use transferrin as a sole source of iron, virulence was maintained, and GFP-activity was stable in vivo. However, FACS sorting and quantification of fluorescent bacteria from the lung did not succeed due to a strong autofluorescence of homogenised lung tissue causing a high signal-to-noise ratio.

In order to monitor the effects of anaerobiosis, outer membrane proteins regulated by ArcA and HlyX (the FNR homologue of *A. pleuropneumoniae*) were chosen as potential targets for an immunohistology-based approach and functionally investigated. The FrpB protein (iron-regulated outer membrane protein B) was chosen as a representative of the HlyX regulon. Despite its name FrpB expression was independent of the global ferric regulator Fur, and growth of the strain was not delayed under anaerobic conditions in comparison to the parent strain. Histopathology of lungs from pigs infected with an *A. pleuropneumoniae frpB* deletion confirmed the strong attenuation of the strain. Functional evaluation revealed that the *A. pleuropneumoniae frpB* deletion was unable to utilise porcine transferrin, the primary iron source of the bacterium and, therefore, the FrpB protein may be the

“missing link” in the bacterial uptake of transferrin-bound iron. Polyclonal sera were raised in rabbits against both, recombinant FrpB protein and bacterial membranes; however, neither of the sera specifically recognized surface-exposed epitopes of the FrpB-protein which would have been a prerequisite for subsequent immunohistology. The Ape51 protein, a homologue to an OmpA-like putative adhesin in *H. ducreyi*, was chosen as a representative of the ArcA regulon and confirmed by 2 dimensional polyacrylamide gel electrophoresis. For characterisation of the protein in vivo and in vitro an isogenic deletion mutant was constructed and confirmed by PCR, PFGE, Southern blot and nucleotide sequence analysis. Growth and survival and adhesion assays did not reveal differences between the *A. pleuropneumoniae* wt and the *A. pleuropneumoniae ape51* deletion mutant. In an infection experiment the mutant was shown to be slightly but not significantly attenuated. Polyclonal sera were raised in rabbits against recombinant Ape51 protein but the serum did not recognise surface-exposed epitopes of the Ape51 protein which would have been a prerequisite for subsequent immunohistology.

7 Zusammenfassung

Gen Regulation in *Actinobacillus pleuropneumoniae* - verschiedene Ansätze zur Bestimmung der Anpassung an Eisenmangel und an sauerstoffunabhängigen Stoffwechsel in vivo

Karla Dreckmann

Der Erreger der Porcinen Pleuropneumonie, *Actinobacillus (A.) pleuropneumoniae* ist ein gram-negatives Bakterium, das für hohe wirtschaftliche Verluste in der industriellen Schweinehaltung verantwortlich ist. Zum besseren Verständnis der Erkrankung wurde die eisen- und sauerstoffabhängige Genregulation unter in vivo und in vitro Bedingungen weiter charakterisiert.

Um Eisenmangel bedingte Effekte zu beobachten wurde ein *A. pleuropneumoniae* Markerstamm konstruiert, der das *gfp* (Grün fluoreszierendes Protein) Gen als Teil des *tbp* (Transferrin bindendes Protein) Operons auf dem Chromosom trägt. Uniforme GFP Expression konnte in vitro durch Eisenrestriktion induziert werden und die Insertion veränderte die Verwendung von Transferrin als Eisenquelle nicht. Die Virulenz des Stammes und die GFP Aktivität blieben in vivo erhalten. Allerdings konnte in infiziertem Gewebe eine FACS Sortierung und die Quantifizierung der fluoreszierenden Bakterien auf Grund starker Autofluoreszenz des homogenisierten Lungengewebes und des dadurch bedingten schlechten Signal-zu-Hintergrund-Verhältnisses nicht quantitativ durchgeführt werden.

Zur Untersuchung der Regulation des anaeroben Stoffwechsels in vivo wurden Außenmembranproteine ausgewählt, die von ArcA oder HlyX (dem FNR Homolog von *A. pleuropneumoniae*) reguliert werden. Diese Proteine wurden als potentielle Ziele für eine Immunhistologie-basierte Herangehensweise weiter untersucht und funktionell charakterisiert. Das FrpB Protein ("iron-regulated outer membrane protein B") wurde als Repräsentant des HlyX Regulons ausgewählt. Es wurde gezeigt, dass FrpB trotz seines Namens unabhängig von der Regulation des globalen Regulators Fur ("ferric uptake regulator") ist, und dass sich das Wachstum einer *frpB* Deletionsmutante unter anaeroben Bedingungen nicht von dem des Wildtyp-

Stammes unterschied. Die histopathologische Untersuchung von Schweinelungen, die mit der *A. pleuropneumoniae frpB* Deletionsmutante infiziert waren bestätigte eine starke Attenuierung des Stammes. Eine funktionelle Auswertung ergab außerdem, dass die *frpB* Deletionsmutante nicht in der Lage ist porcines Transferrin zu verwenden; dieses ist jedoch die primäre Eisenquelle des Bakteriums in vivo. Das FrpB könnte somit das fehlende Bindeglied in der bakteriellen Eisenaufnahme von Transferrin-gebundenem Eisen sein. Polyklonale Seren gegen rekombinantes FrpB und gegen Bakterienmembranen wurden im Kaninchen hergestellt. Jedoch erkannte keines dieser Seren die Oberflächen-exponierten Epitope des FrpB Proteins. Das wäre jedoch die Grundvoraussetzung für eine anschließende Verwendung der Seren in der Immunhistochemie gewesen.

Das Ape51 Protein, ein Homolog eines OmpA-ähnlichen, putativen Adhäsins in *H. ducreyi* wurde als Repräsentant des ArcA Regulons ausgewählt und in der Zweidimensionalen Polyacrylamid Gelelektrophorese bestätigt. Für die in vivo und in vitro Charakterisierung des Proteins wurde eine Deletionsmutante konstruiert und mittels PCR, Pulsfeldgelelektrophorese, Southern Blot und DNA Sequenzierung überprüft. Wachstums- und Überlebenskurven zeigten keine Unterschiede zwischen dem *A. pleuropneumoniae* Wildtyp und der *ape51* Deletionsmutante. In einem Infektionsversuch zeigte sich die Mutante leicht, aber nicht statistisch signifikant attenuiert. Polyklonale Kaninchenserum gegen rekombinantes Ape51 erkannten Oberflächen-exponierte Epitope von Ape51 nicht spezifisch. Das wäre jedoch die Grundvoraussetzung für eine anschließende Verwendung der Seren in der Immunhistochemie gewesen.

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9 Appendix

9.1 Raw data of the animal experiments

Table 8 Raw data for challenge experiments with *A. pleuropneumoniae* wt, *A. pleuropneumoniae* Δ *ape51* and the *A. pleuropneumoniae* GFP marker strain.

ear tag/ animal ID	group	total clinical score	lung score	ELISA ApxIIA	reisolation from organ samples			
					lung, pn eu mo nic	lung, intac t	ly mp h no de	ton sil
6125 ^c	GFP marker strain	3	0	-	-	+++	++	++
6126 ^c	GFP marker strain	3	0	-	-	+++	+	++
6177 ^c	GFP marker strain	3	0	-	-	+++	++	+
6132 ^c	GFP marker strain	0	0	-	-	+	+	+
1593 ^c	Δ <i>ape51</i>	20	25.8	0	-	+++	++	-
6028 ^a	Δ <i>ape51</i>	0	0	1	-	-	-	++
6035 ^a	Δ <i>ape51</i>	0	0	0	-	-	-	++
1509 ^a	Δ <i>ape51</i>	0	0	1	-	-	-	+
6026 ^a	Δ <i>ape51</i>	0	0	0	-	-	-	++
1583 ^a	Δ <i>ape51</i>	0	0	0	-	-	-	-
1597 ^a	Δ <i>ape51</i>	0	0	0	-	-	-	++
1500 ^a	Δ <i>ape51</i>	2	9.8	1	-	++	+	-
8858 ^a	Δ <i>ape51</i>	0	4,6	0	+++	+	+	++
8745 ^a	Δ <i>ape51</i>	0	0	0	-	-	+	-
8750 ^a	Δ <i>ape51</i>	0	0	0	-	-	-	-
8784 ^a	Δ <i>ape51</i>	0	0	0	-	-	-	-
8730 ^b	Δ <i>ape51</i>	0	0	1	-	-	-	-
8741 ^b	Δ <i>ape51</i>	0	0	1	-	-	-	-
8753 ^b	Δ <i>ape51</i>	0	0	1	-	-	-	-
8747 ^b	Δ <i>ape51</i>	0	0	1	-	-	-	-
1571 ^a	wild type	7	5	2	+++	+	+	+
6085 ^a	wild type	0	0	0	-	-	-	+

ear tag/ animal ID	group	total clinical score	lung score	ELISA ApxIIA	reisolation from organ samples			
					lung, pneumonic	lung, intact	lymph node	tonsil
1507 ^a	wild type	3	11.9	6	++	-	+	+
1554 ^a	wild type	0	0	0	-	-	-	-
1551 ^a	wild type	0	0	0	-	-	-	+
6045 ^a	wild type	0	8.8	1	-	+	++	+
1582 ^a	wild type	0	0	0	-	-	-	-
1516 ^a	wild type	0	0	0	-	-	-	-
8775 ^a	wild type	0	0	0	-	-	-	-
8777 ^a	wild type	2	12.5	1	++	+	++	+
8785 ^a	wild type	0	0	0	-	-	-	-
8793 ^a	wild type	2	7.4	0	-	+	+	+
8776 ^b	wild type	0	0	0	-	-	-	-
8755 ^b	wild type	0	0	0	-	-	-	-
8780 ^b	wild type	0	0	0	-	-	-	-
8859 ^b	wild type	0	8.4	80	+++	-	++	++

^a euthanised 7 days post infection

^b euthanised 21 days post infection

^c euthanised 1 day post infection because of severe clinical symptoms

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