Construction and characterization of genetically defined metabolic mutants of *Actinobacillus pleuropneumoniae*

INAUGURAL-DISSERTATION
zur Erlangung des Grades
eines Doktors der Veterinärmedizin
(Dr. med. vet.)
durch die Tierärztliche Hochschule Hannover

Vorgelegt von

**Kyaw Sunn**
**Yamethin, Myanmar**

Hannover 2003
Wissenschaftliche Betreuung: Univ.-Prof. Dr. G.-F. Gerlach

1. Gutachter: Univ.-Prof. Dr. G.-F. Gerlach
2. Gutachter: Univ.-Prof. Dr. V. Moennig

Tag der mündlichen Prüfung: 21.11.2003

Gefördert durch ein Stipendium des Deutschen Akademischen Austauschdienstes
My parents
A part of this study was presented:

SUNN, K., G.-F. GERLACH, N. BALTES (2003):
Virulenz isogener [NiFe] Hydrogenase 2 Mutanten von *Actinobacillus pleuropneumoniae*
25. Kongress der DVG, 3-4 April 2003, Berlin
Table of contents

1 Introduction .................................................................................................................. 11

2 Literature review ............................................................................................................ 12

2.1 *Actinobacillus pleuropneumoniae* ........................................................................... 12
  2.1.1 Taxonomy .......................................................................................................... 12
  2.1.2 Epidemiology .................................................................................................... 13
  2.1.3 Immunity and vaccination ................................................................................. 14
  2.1.4 Role of virulence factors .................................................................................... 16
    2.1.4.1 Capsule ....................................................................................................... 16
    2.1.4.2 Lipopolysaccharide (LPS) .......................................................................... 16
    2.1.4.3 Transferrin binding proteins .................................................................... 17
    2.1.4.4 Apx Toxins ................................................................................................. 18
    2.1.4.5 Urease ........................................................................................................ 19
    2.1.4.6 Other virulence factors .............................................................................. 20
  2.1.5 Molecular biology of NiFe hydrogenases ......................................................... 20
    2.1.5.1 Organization and structural genes of NiFe hydrogenases ...................... 20
    2.1.5.2 Virulence and NiFe hydrogenase 2 ......................................................... 22
  2.2 Bacterial fucose metabolism ................................................................................... 23
    2.2.1 Organization and structural genes ................................................................... 23
    2.2.2 Fucose and bacterial virulence ................................................................. 24

3 Materials and methods ................................................................................................. 26

3.1 Bacterial cultures ..................................................................................................... 26
  3.1.1 Bacterial strains .............................................................................................. 26
  3.1.2 Media and growth condition .......................................................................... 26

3.2 Isolation of DNA ...................................................................................................... 27
  3.2.1 Isolation of plasmid DNA .............................................................................. 27
    3.2.1.1 Minipreparation of plasmid DNA ....................................................... 27
3.2.1.2 Preparation of plasmid DNA (Midi-Prep with JetStar® Kit).................................................28
3.2.2 Isolation of chromosomal DNA from \textit{A. pleuropneumoniae} ...........................................29

3.3 DNA purification..................................................................................................................33
3.3.1 DNA purification from TAE-agarose gels by adsorption to a silica matrix........33

3.4 Plasmid construction.........................................................................................................34
3.4.1 Digestion of DNA with restriction endonucleases .......................................................34
3.4.2 Agarose gel electrophoresis..........................................................................................34
3.4.3 Generation of blunt-end DNA by 5' overhang fill-in ..................................................35
3.4.4 Generation of blunt-end DNA by 3' overhang removal ..............................................35
3.4.5 Alkaline phosphatase treatment..................................................................................36
3.4.6 Ligation.........................................................................................................................36

3.5 Transformation..................................................................................................................36
3.5.1 Preparation of \textit{E. coli} chemical competent cells for transformation ..................36
3.5.2 Transformation of \textit{E. coli} by heat shock.................................................................37

3.6 Transconjugation from \textit{E. coli} to \textit{A. pleuropneumoniae} by filter mating technique ....38

3.7 Sucrose counterselection .................................................................................................38

3.8 Polymerase chain reaction (PCR)......................................................................................39

3.9 Southern blot.....................................................................................................................44
3.9.1 Southern blotting........................................................................................................44
3.9.2 Labelling of probe with $\alpha^{32}$P-dCTP using the random priming method ............45
3.9.3 Southern blot hybridization.......................................................................................45

3.10 Colony blot......................................................................................................................46
3.10.1 Colony blotting..........................................................................................................46
3.10.2 Colony blot hybridization.........................................................................................47

3.11 Pulsed-field gel electrophoresis (PFGE)..........................................................................47
3.11.1 Preparation of total DNA of \textit{A. pleuropneumoniae}-embedded agarose plugs for pulsed-field gel electrophoresis .........................................................................................47
3.11.2 Restriction of DNA embedded in agarose gel .........................................................49
3.11.3 Pulsed-field gel electrophoresis................................................................................49
3.12 Nucleotide sequencing and sequence analysis .............................................................50
3.13 Challenge experiment in the pig ...................................................................................50
  3.13.1 Challenge experiment timeline .............................................................................50
  3.13.2 Origin and housing of the animals .........................................................................51
  3.13.3 Aerosol infection chamber ....................................................................................51
  3.13.4 Preparation of bacteria for aerosolization .............................................................51
  3.13.5 Aerosol infection ..................................................................................................52
    3.13.5.1 Surveillance of the animals during the experiment ........................................52
  3.13.6 Bronchoalveolar lavage fluid (BALF) ..................................................................52
    3.13.6.1 Bacteriological examination of BALF ............................................................53
  3.13.7 Postmortem examination ......................................................................................53
    3.13.7.1 Determination of lung lesion scores ...............................................................53
    3.13.7.2 Bacteriological examination of organ samples ...............................................54
  3.13.8 Enzyme Linked Immunosorbent Assay (ELISA) .................................................54
  3.13.9 Statistics ...............................................................................................................55

4 Results ...........................................................................................................................56
  4.1 The *A. pleuropneumoniae* [NiFe] hydrogenase 2 gene (*hybB*) .........................56
    4.1.1 Sequence analysis .................................................................................................56
    4.1.2 Construction and characterization of an isogenic *A. pleuropneumoniae* [NiFe] hydrogenase 2 mutant ...............................................................56
      4.1.2.1 Construction of plasmid pHYB603 for the introduction of a [NiFe] hydrogenase 2 deletion into *A. pleuropneumoniae* AP76 .................................56
      4.1.2.2 Construction of an isogenic *A. pleuropneumoniae* mutant deficient in [NiFe] hydrogenase 2 ...............................................................................................57
      4.1.2.3 Analysis of isogenic [NiFe] hydrogenase 2 deletion mutants ..........................57
    4.1.3 Role of the [NiFe] hydrogenase 2 in an *A. pleuropneumoniae* aerosol infection experiment ..................................................................................................................61
      4.1.3.1 Post mortem examination .................................................................................61
      4.1.3.2 Humoral immune response ...........................................................................61
  4.2 The *A. pleuropneumoniae* L-1,2 Propanediol Oxidoreductase gene (*fucO*) ...........66
4.2.1 Sequence analysis .........................................................................................................................66
4.2.2 Construction and characterization of an isogenic *A. pleuropneumoniae* L-1,2 propanediol oxidoreductase mutant........................................................................................................66
   4.2.2.1 Construction of plasmid pFUO604 for the introduction of a L-1,2 propanediol oxidoreductase deletion into *A. pleuropneumoniae* AP76 ........................................... 66
   4.2.2.2 Construction of an isogenic *A. pleuropneumoniae* mutant deficient in L-1,2 Propanediol Oxidoreductase ........................................................................................................67
   4.2.2.3 Analysis of isogenic L-1,2 propanediol oxidoreductase deletion mutants ...... 67
4.2.3 Role of L-1,2 propanediol oxidoreductase in an *A. pleuropneumoniae* aerosol infection experiment ............................................................................................................................71
   4.2.3.1 Post mortem examination ........................................................................................................71
   4.2.3.2 Systemic immune response .......................................................................................................72

5 Discussion .............................................................................................................................................76
6 Summary/Zusammenfassung ......................................................................................................................80
7 References ................................................................................................................................................84
8 Appendix ................................................................................................................................................105
   8.1 Chemicals ........................................................................................................................................105
   8.2 Sequence of *A. pleuropneumoniae hybB* .....................................................................................107
   8.3 Sequence of *A. pleuropneumoniae fucA, fucP* and *fucO* ..............................................................110
   8.4 Animal experiment ............................................................................................................................123
   8.5 Index of figures ................................................................................................................................125
   8.6 Index of tables ................................................................................................................................125
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. bidest.</td>
<td>Aqua bidestillata</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td><em>Actinobacillus pleuropneumoniae</em></td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>cfu</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytidine triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanosine triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxy nucleoside triphosphate</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>et al.</td>
<td>et alii</td>
</tr>
<tr>
<td>Fig.</td>
<td>Figure</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>IVX</td>
<td>IsoVitaleX</td>
</tr>
<tr>
<td>kb</td>
<td>kilo base pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>OD&lt;sub&gt;xxx&lt;/sub&gt;</td>
<td>optical density at xxx nanometre</td>
</tr>
<tr>
<td>OmL1A</td>
<td>outer membrane lipoprotein A</td>
</tr>
<tr>
<td>PPLO</td>
<td>pleuropneumonia-like organism</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>rpm</td>
<td>rounds per minute</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
</tbody>
</table>
1 Introduction

*Actinobacillus (A.) pleuropneumoniae* is a highly infectious porcine respiratory tract pathogen. The disease caused by this bacterium is encountered worldwide and frequently causes severe losses in infected herds. Infected animals develop disease symptoms ranging from acute fibrinous pneumonia and pleuritis with high mortality to chronic lung lesions resulting in reduced growth rates. Pigs that survive an infection develop a protective immune response but can still be carriers of the pathogen. The rapid onset and severity of disease means that losses may occur before antibiotic therapy has time to become effective. Attempts to immunize against *A. pleuropneumoniae* disease have been hampered by the occurrence of two biotypes with at least 12 serotypes and the lack of reliable cross-protection between serotypes upon immunization with conventional formaldehyde inactivated bacterin vaccines. The construction of isogenic mutants, which cannot persist in the host over an extended period, may solve that problem by inducing the development of a cross-protective immune response.

Animals that survive an infection remain carriers of the pathogen for weeks or months with *A. pleuropneumoniae* persisting in the pigs' tonsils and lungs. Under these conditions, the pathogen encounters anaerobic conditions, and therefore, it was hypothesized that impairment of anaerobic metabolism might reduce the pathogen’s multiplication rate and thus result in attenuation or reduced persistence in an aerosol infection model.

BALTES (2002) identified the [NiFe] hydrogenase 2 subunit (HybB) and the L-fuculose 1-phosphate aldolase (FucA), a member of the fucose regulon, in the differential screening of an *A. pleuropneumoniae* cDNA library selecting for cDNAs primarily expressed under *in vivo*-like conditions. For further characterization, larger fragments containing entire genes had to be obtained by screening a genomic library of *A. pleuropneumoniae* by using the cDNA fragments as probe. The gene encoding the [NiFe] hydrogenase 2 subunit (*hybB*) and the gene encoding the L-1,2 propanediol oxidoreductase gene (*fucO*) which is part of the fucose regulon were chosen for the construction of isogenic deletion mutants; subsequent assessment of virulence followed because these genes are expressed under anaerobic conditions and therefore were hypothesized to be involved in bacterial persistence in necrotic lesions.
2 Literature review

2.1 *Actinobacillus pleuropneumoniae*

2.1.1 Taxonomy

*Actinobacillus (A.) pleuropneumoniae* is a member of *Haemophilus-Actinobacillus-Pasteurella* (HAP) group in the family *Pasteurellaceae*. *Actinobacillus pleuropneumoniae* is a gram-negative rod, non-motile, non-spore forming, hemolytic, and urease positive. It grows in a nicotinamide adenine dinucleotide (NAD)-dependent manner on sheep blood agar with hemolysis. *Actinobacillus pleuropneumoniae* enhances the hemolysis caused by the β-hemolysin of *Staphylococcus aureus* in a CAMP test (named after CHRISTIE, ATKINS, and MUNCH-PETERSEN; MANNHEIM 2002). The first field isolates of *A. pleuropneumoniae* were reported in the early 1960's in Great Britain, California, and Argentina (MATHEW and PATTISON 1961; SHOPE 1964). At the time the isolates were typed as one of three species: *Haemophilus (H.) parainfluenzae*, *H. parahaemolyticus*, and *H. pleuropneumoniae*. In 1983, DNA hybridization studies indicated that there was no significant homology between *H. pleuropneumoniae* and *H. influenzae*. However, *H. pleuropneumoniae* and *Actinobacillus lignieresii* showed significant relatedness. This resulted in a proposal to change the genus from *Haemophilus* to *Actinobacillus*.

*Actinobacillus pleuropneumoniae* isolates that require NAD for growth are designated as biotype 1 while *A. pleuropneumoniae* isolates that are NAD independent are designated as biotype 2 (POHL et al. 1983; NICOLET 1992; NIELSEN et al. 1997). On the basis of capsular and lipopolysaccharide antigens, 12 serotypes of *A. pleuropneumoniae* have been defined; however cross-reactions are common (NICOLET 1988). Cross reactivity has been reported to occur between serotypes 1, 9 and 11, 3, 6 and 8, and 4 and 7 (NIELSEN 1985; MITTAL et al. 1988; MITTAL 1990). Serological cross-reactions among different serotypes coincide with the structural similarity of O-antigens. *Actinobacillus pleuropneumoniae* serotypes 1 and 5 are further differentiated into 1a and 1b, and 5a and 5b, respectively, based on minor differences in the polysaccharide structures (PERRY et al. 1990; JOLIE et al. 1994).
2.1.2 Epidemiology

*A. pleuropneumoniae* is the causative agent of porcine pleuropneumonia and was first identified in 1957 in Great Britain by PATTISON and coworkers (MATHEW and PATTISON 1961; NICOLET 1992). The organism is highly host specific for pigs, but has occasionally been isolated from lambs (NIELSEN 1986). Serotypes of *A. pleuropneumoniae* are generally distributed by geographic location; serotype 1, 5, and 7 are most commonly found in the United States. In Canada, serotypes 1, 3, and 5 are isolated frequently, while serotypes 1, 2, 5, 7 and 9 are important in Europe (MULKS and BUYSE 1992; HENNESSY et al. 1993).

The primary route of infection is by infected droplets over a short distance by shared air space or by direct contact with infected pigs (TORREMORELL et al. 1997). In acute outbreaks the disease agent may be transmitted from one pen to another, suggesting that transmission by aerosol or by farm personnel carrying contaminated exudate of infected pigs is also possible (NICOLET 1992). Subclinically infected carrier pigs are by far the most common means of transmitting *A. pleuropneumoniae* between herds. Clothing, vehicles and, possibly, flies are of secondary importance because the organism persists in the environment for a short period of time only. Pigs are the only known carriers of *A. pleuropneumoniae* (FENWICK and HENRY 1994).

Porcine pleuropneumonia caused by *A. pleuropneumoniae* affects pigs of all ages, although 10-16 weeks old pigs are most susceptible (FENWICK and HENRY 1994). Characteristic symptoms of the disease range from acute fibrinous pneumonia and pleuritis with high mortality to nearly asymptomatic colonization by the bacterium (MACINNES et al. 1988). In the chronic state, lung lesions present themselves as sequestered abscesses with persistent pleural adhesions (LIGGETT et al. 1987; DIDIER et al. 2002). Pigs that survive infection can still be carriers of the pathogen. Carrier pigs have chronically colonized tonsils, nasal cavities, and focal pulmonary lesions. Clinically healthy carrier pigs harboring the bacterium have been demonstrated to pose a continuous threat to non-infected animals of the herd under field conditions and, therefore, a herd once infected remains infected (KUME et al. 1984). Carrier pigs have a reduced economic performance due to decreased feed conversion rates, low weaning rates, and reduced market value (MOLLER et al. 1993).
Outbreaks of acute disease caused by *A. pleuropneumoniae* are usually associated with an intensive production scheme. Outbreaks with high mortality often occur shortly after weaning (NICOLET 1992). Deaths occur mostly within the first four days after infection (SEBUNYA and SAUNDERS 1983).

2.1.3 Immunity and vaccination

The nature of protective immunity to porcine pleuropneumonia is not clear (RYCROFT and GARSIDE 2000). NIELSEN (1979, 1985) reported cross-protection between serotypes after natural infection, although this has been challenged by subsequent experiments, which found some but not universal cross-protection (CRUJISEN et al. 1995; HAESEBROUCK et al. 1997). The humoral immune response is thought to be a key part in the host protection against *A. pleuropneumoniae*, with IgG playing a major role (INZANA et al. 1988; BOSSE et al. 1992; JANSEN et al. 1994). Circulating antibodies can be detected with the complement fixation test within 10 days after experimental infection. Antibody titers reach a maximum 3 to 4 weeks after infection and persist for many months (BOSSE et al. 1992; JANSEN et al. 1994). Immune sows confer passive immunity on their offsprings. Such colostral antibodies may persist about 5-12 weeks, but the protection does not seem to last for more than 3 weeks (NICOLET 1992). Passive transfer of porcine immune serum produces protection against *A. pleuropneumoniae* (BOSSE et al. 1992). Neutralizing antibodies to Apx toxins are present in convalescent serum and are associated with protection against pleuropneumonia (DEVENISH et al. 1990; CRUJISEN et al. 1995).

The contribution of a cell-mediated immune response (CMI) is not as well defined, but FURESZ et al. (1997) reported that a strong delayed type hypersensitivity response (as a measure of CMI) and antibody responses were associated with protection.

Pigs surviving a natural infection with *A. pleuropneumoniae* develop immunity to the organism and are protected from clinical symptoms in further infections with homologous and heterologous serotypes (NIELSEN 1984; INZANA 1991). However, the antigens that produce this solid protective immunity have not been clearly identified, and this cross-protection has not yet been achieved with vaccines produced commercially. To date, commercial vaccines are based on whole inactivated bacteria or subunits, which include
capsular polysaccharide, lipopolysaccharide, and outer membrane proteins of various combinations of serotypes 1, 2, 5, 7 and 9 of *A. pleuropneumoniae*. Results obtained with these vaccines have offered, at least, homologous protection against the serotypes which were used to prepare the vaccine material (NICOLET 1992; FENWICK and HENRY 1994; HAESEBROUCK et al. 1997; CHIERS et al. 1998).

The production of toxin(Apx)-neutralizing antibodies during infection plays a major role in the induction of protective immunity to *A. pleuropneumoniae* reinfection (INZANA et al. 1991). Among the different serotypes of *A. pleuropneumoniae*, four of these exotoxins have been described, and each serotype produces either two or three of them (KAMP et al. 1991; FREY et al. 1993; SCHALLER et al. 1999). Although antibodies against Apx toxins seem to be most important in protection (ROSSI-CAMPOS et al. 1992; KAMP et al. 1997), antibodies induced by any single antigen do not protect completely against colonization and infection (HAESEBROUCK et al. 1997). CHIERS et al. (1998) also demonstrated that a commercial subunit vaccine containing all three Apx toxins could not prevent the development of lung lesion. GOETHE et al. (2000) have produced a subunit vaccine from *A. pleuropneumoniae* serotype 2 and 9 cultures grown under iron-deficient conditions and tested it in an animal experiment. Immunized pigs showed strongly reduced clinical symptoms, but the vaccine did not prevent colonization.

The bacterial ghost system is a novel vaccine delivery system. Bacterial ghosts are bacterial cell envelopes devoid of cytoplasmic contents. *A. pleuropneumoniae* ghost formation is achieved by the expression of a cloned bacteriophage PhiX174 lysis gene E resulting in transmembrane tunnel formation and loss of the cytoplasmic contents including DNA through this tunnel (SZOSTAK et al. 1996). Experimental intramuscular vaccination of *A. pleuropneumoniae* serotype 9 ghosts was found to be associated with complete protection against homologous challenge and prevented colonization (HENSEL et al. 2000).

Initial experiments with attenuated live vaccines showed that insertion mutants requiring riboflavin for growth could provide significant protection against mortality and reduction in lung damage and clinical signs of pleuropneumonia (FULLER et al. 2000). However, this vaccine strain carries an antibiotic marker which is prohibitive for licensing. Recently, TONPITAK et al. (2002) have successfully constructed an isogenic *A. pleuropneumoniae*
serotype 2 double mutant without antibiotic marker by using a single step transconjugation system. The \textit{ureC} and \textit{apxIIA} genes were deleted in this mutant. They performed protection experiments using this mutant as a live vaccine in a single aerosol immunization. The results showed that the mutant strain is highly attenuated and immunized pigs were protected significantly from colonization of the lungs. Furthermore, this mutant strain facilitates the serological discrimination of immunized and naturally infected herds.

2.1.4 Role of virulence factors

There are a number of factors that significantly contribute to the pathogenic properties of \textit{A. pleuropneumoniae}. These factors may also play a role for survival of the bacterium in the host. The following is a short summary of these virulence factors.

2.1.4.1 Capsule

Capsules are found in all strains of \textit{A. pleuropneumoniae}. All serotypes are distinguished by capsular polysaccharides (PERRY et al. 1990). The main role of the capsule appears to be the protection of the pathogen; encapsulated \textit{A. pleuropneumoniae} is protected from complement-mediated killing in the presence and absence of capsule specific antibodies (INZANA et al. 1988). Thus, capsule-deficient strains of \textit{A. pleuropneumoniae} serotype 5 were effectively killed in the presence of both, normal and immune serum, whereas encapsulated strains were not (INZANA et al. 1988; WARD and INZANA 1994; WARD et al. 1998).

The importance of \textit{A. pleuropneumoniae} capsule expression for virulence has been assessed in experimental infections in pigs using nonencapsulated mutants generated by gene replacement in a serotype 5a strain (WARD et al. 1998) and transposon mutagenesis of a serotype 1 strain (RIOUX et al. 1999). In both cases, the nonencapsulated mutants were severely disabled compared to their encapsulated parent strains in their ability to cause lung lesions and mortality in pigs.

2.1.4.2 Lipopolysaccharide (LPS)

LPS is known to play a major role in the pathogenesis of infections with gram-negative bacteria, including porcine pleuropneumonia. LPS consists of lipid A, a core region of
common sugars, and an O-polysaccharide side chain (BYRD and KADIS 1989). Although pure LPS of *A. pleuropneumoniae* has the potential to cause damage to lung tissue, the damage differs from the hemorrhagic and necrotic lung lesions found in typical cases of *A. pleuropneumoniae* infection (UDEZE et al. 1987). Therefore, it has been hypothesized that LPS and exotoxin are able to interact thereby intensifying the damaging effects of *A. pleuropneumoniae* (INZANA 1991; FENWICK and HENRY 1994). In addition, LPS may play a role in adherence of *A. pleuropneumoniae*; this was first suggested following the observation that strains expressing smooth type LPS adhered to cultured porcine tracheal rings in higher numbers than those expressing LPS of the semirough type (BELANGER et al. 1990). Subsequently, it was shown that bacterial adherence could be inhibited by purified LPS (PARADIS et al. 1994).

**2.1.4.3 Transferrin binding proteins**

Iron is essential for bacterial growth. However, it is not readily available in the extracellular environment of the host due to complexation by the host glycoproteins, transferrin and lactoferrin. Several *Actinobacillus*, *Haemophilus* and *Pasteurella* species have been shown to possess a mechanism of iron acquisition from transferrin (NIVEN et al. 1989). Two distinct transferrin binding proteins are produced under iron restricted growth conditions (GONZALEZ et al., 1990; RICARD et al. 1999). The TbpA protein (also known as Tbp1 or TfbB) has an approximate molecular mass of 100 kDa and likely forms a transmembrane channel for transport of iron across the outer membrane (GONZALEZ et al. 1995; DABAN et al. 1996; WILKE et al. 1997). The TbpB protein (also known as Tbp2 or TfbA) has an approximate molecular mass of 60 kDa and is a lipoprotein anchored in the outer membrane (GERLACH et al. 1992; GONZALEZ et al. 1995; FULLER et al. 1998). There is evidence that an interaction between these proteins is required for optimal utilization of transferrin as a sole source of iron (GONZALEZ et al. 1995; FULLER et al. 1998; LITT et al. 2000). In addition, GERLACH et al. (1992) showed that TbpB is also able to bind hemin but not hemoglobin. This binding specificity has not been tested for TbpA.

In *A. pleuropneumoniae*, *tbpA* and *tbpB* genes are transcriptionally linked to the *exbBD* genes (TOPNPITAK et al. 2000). The ExbB and ExbD proteins form an inner membrane protein complex that, in association with TonB, provides energy to high affinity receptors enabling
transport of iron across the outer membrane (MOECK et al. 1998). A partial open reading frame sharing homology to the neisserial TonB on the protein level was found upstream of $exbB$ and may be located on the same transcript driven by a single iron-regulated promotor (TONPITAK et al. 2000). TONPITAK et al. (2000) demonstrated that expression of ExbB and ExbD is essential for utilization of transferrin-bound iron by $A. pleuropneumoniae$. Furthermore, both $exbB$ and $tonB$ transposon mutants of $A. pleuropneumoniae$ are avirulent, demonstrating the central role of this complex in iron uptake (FULLER et al. 2000).

2.1.4.4 Apx Toxins

The hemolytic and cytotoxic characteristics that have been associated with $A. pleuropneumoniae$ have been attributed to at least four toxin proteins. Early analyses of the proteins have identified them as members of the RTX-toxin family (LALONDE et al. 1989). These toxins have been named Apx-toxins (for $A. pleuropneumoniae$ RTX-toxins) and are designated as ApxI, ApxII, ApxIII (FREY et al. 1993) and ApxIV (SCHALLER et al. 1999). Each toxin varies in its hemolytic and cytotoxic activities, and their presence in $A. pleuropneumoniae$ strains depends on its serotype (FREY et al. 1996).

ApxI is a strongly hemolytic and cytotoxic protein with a molecular mass of 105-110 kDa (FREY and NICOLET 1988). It is produced and secreted by $A. pleuropneumoniae$ serotypes 1, 5, 9, 10, and 11 (FREY and NICOLET 1990; KAMP et al. 1994) and encoded by the $apxICABD$ operon (GYGI et al. 1992; JANSEN et al. 1993). Strains that produce ApxI tend to be highly virulent, supporting evidence that toxin activity is related to the virulence of $A. pleuropneumoniae$ (FREY and NICOLET 1990; KAMP et al. 1991).

ApxII is weakly hemolytic and weakly cytotoxic and has a molecular mass of 103-105 kDa (FREY and NICOLET 1988). All $A. pleuropneumoniae$ serotypes with the exception of serotype 10 produce and secrete ApxII (KAMP et al. 1991; 1994). However, the $apxII$ operon does not contain any genes that are required for secretion (FREY 1995). Apparently, the secretion of ApxII depends on the $apxIBD$ genes, which are found in all $A. pleuropneumoniae$ serotypes with the exception of serotype 3 (FREY 1995).
ApxIII is not hemolytic but is strongly cytotoxic with a molecular mass of 120 kDa (KAMP et al. 1991; RYCROFT et al. 1991). The protein is produced and secreted by *A. pleuropneumoniae* serotypes 2, 3, 4, 6, and 8 (KAMP et al. 1991; FREY et al. 1993). The operon encoding for ApxIII consists of the genes *apxIIICABD* and has an arrangement analogous to that of the *apxI* operon (CHANG et al. 1993).

More recently, a fourth RTX-toxin (ApxIV) was reported to be produced by all serotypes of *A. pleuropneumoniae* (SCHALLER et al. 1999). This toxin is antigenically distinct from the other Apx toxins and is only produced in vivo.

Apx toxins play a role in evasion of the host's first line of defense; at high concentration of RTX toxins causes the formation of pores in the membrane of phagocytic and other target cells, resulting in osmotic swelling and cell death. Furthermore, there are indications that the toxins are involved in the development of lesions; they are toxic for endothelial cells (SEREBRIN et al. 1991) and, at low concentrations, provoke an oxidative burst in macrophages (DOM et al. 1992b) and neutrophils (DOM et al., 1992a) resulting in excessive production of oxygen radicals which can have deleterious effects on host cells. Moreover, purified recombinant Apx toxins were able to cause lung lesions upon endobronchial instillation, whereas mutant strains, which are unable to produce Apx toxins, did not induce lesions (KAMP et al. 1993; STOCKHOFE-ZURWIEDEN et al. 1996). Use of transposon mutagenesis (TASCON et al. 1994) and complementation experiments (REIMER et al. 1995) proved that Apx toxins are essential in the pathogenesis of porcine pleuropneumonia. In addition, mutants of serotype 1 and 5 strains, devoid of ApxI but still producing ApxII, also caused typical severe clinical disease and lung lesions (TASCON et al. 1994; REIMER et al. 1995).

### 2.1.4.5 Urease

The urease enzyme catalyzes the hydrolysis of urea into ammonia and carbon dioxide and is known to be a virulence factor in certain gastrointestinal and urinary tract pathogens (COLLINS et al. 1993). Although all *A. pleuropneumoniae* field isolates are strongly urease positive, a spontaneously urease negative *A. pleuropneumoniae* wild type strain has been isolated from a case of acute pneumonia (BLANCHARD et al. 1993). A clinical challenge
trial showed that, at low challenge doses, urease negative mutants are unable to establish infection (BOSSE and MACINNES 2000). BALTES et al. (2001) reported that urease activity may cause sufficient impairment of the local immune response to slightly improve the persistence of the urease positive *A. pleuropneumoniae* parent strains in comparison to urease negative mutants.

### 2.1.4.6 Other virulence factors

An antigenic protease is produced *in vivo* by all serotypes of *A. pleuropneumoniae*. NEGRETE-ABSCAL et al. (1994) reported that proteases could have a role in the disease and in the immune response of pigs infected with *A. pleuropneumoniae*. GARCAI-CUELLAR et al. (2000) showed that protease secreted from *A. pleuropneumoniae* degrades porcine hemoglobin, IgA and gelatin.

*A. pleuropneumoniae* contains a periplasmic Copper- and Zinc-cofactored superoxide dismutase ([Cu,Zn]-SOD or SodC) that protects *A. pleuropneumoniae* against oxygen radicals in vitro (KROLL et al. 1995; LANGFORD et al. 1996). SHEEHAN et al. (2000) showed that a [Cu,Zn]-SOD-deficient *A. pleuropneumoniae* serotype 1 mutant remained fully virulent in experimental pulmonary infection in pigs.

The *ohr* gene, encoding an organic hydroperoxide reductase, was recently identified (SHEA and MULKS 2002) and could play a role in the detoxification of organic peroxides generated during infection.

### 2.2 Molecular biology of [NiFe] hydrogenases

#### 2.2.1 Organization and structural genes of [NiFe] hydrogenases

Hydrogenase enzymes are widely distributed among microorganisms and are involved in many biological processes where hydrogen is consumed or generated. Hydrogenases catalyze the interconversion of molecular hydrogen into protons and electrons according to the reaction: $\text{H}_2 \leftrightarrow 2\text{H}^+ + 2\text{e}^-$. Hydrogenases are metalloenzymes that can be differentiated into two main types by the metal content: iron (Fe) only hydrogenase and nickel and iron [NiFe]
hydrogenases. [NiFe] hydrogenases contain one nickel (Ni) and one iron (Fe) atom at their active site (VOLBEDA et al. 1995).

*Escherichia* (*E.*) *coli* synthesizes at least three [NiFe] hydrogenases. Whereas hydrogenase 3 is responsible for formate-dependent H₂ evolution (BÖHM et al. 1990), both hydrogenase 1 and hydrogenase 2 are H₂-uptake enzymes differing in their immunological, genetic and physicochemical properties (BALLANTINE and BOXER 1985; MENON et al. 1991, 1994).

Hydrogenase 1 is a transmembrane protein that is purified as a heterodimer consisting of a 64 kDa large subunit and a 35 kDa small subunit (SAWERS and BOXER 1985). The *hya* operon encoding hydrogenase 1 has been cloned, sequenced and shown to comprise six open reading frames, *hyaABCDEF* (MENON et al. 1990). The *hyaA* gene encodes a 40.6 kDa protein possessing a long amino-terminal signal sequence. This sequence is removed during biosynthesis to leave the mature membranebound 35 kDa subunit; the nickel-containing large subunit is encoded by *hyaB*. The role of the remaining gene products remains unclear. However, the *HyaC* protein has some sequence identity with b-type cytochromes, most notably that of the hydrogenase of *Wollinella succinogenes* (DROSS et al. 1992).

Hydrogenase 2 was shown to participate in H₂-dependent fumarate reduction (BALLANTINE and BOXER 1985; MENON et al. 1994). The DNA sequence of the structural operon *hybABCDEFG* of *E. coli* hydrogenase 2 was established by MENON et al. (1994). The purified enzyme was obtained from the membrane fraction following trypsin-dependent release and characterized as a soluble tetramer comprising two large subunits of 61 kDa and two small subunits of 31 kDa (BALLANTINE and BOXER 1985). It was established that the *hybC* gene encodes the large subunit of the enzyme following the sequencing of several peptides derived from the large subunit (MENON et al. 1994). However, at that time, it was only suggested that the small subunit was encoded by *hybA*. Later studies by SARGENT and coworkers (1998) clearly established that the small subunit of the enzyme is not encoded by *hybA* but by a previously overlooked gene, termed as *hybO* that, in the operon, is located immediately upstream of *hybA*. HybB, like the *hyaC* gene product, has sequence similarity to b-type cytochromes.
Hydrogenase 3 is a component of the formate hydrogen lyase complex (FHL complex) that accounts for the hydrogen production under fermentative conditions (BÖHM et al. 1990). Hydrogenase 3 is encoded by the $hyc$ operon, which is composed of nine open reading frames $hycABCDEFGHI$ (BÖHM et al. 1990; ROSSMANN et al. 1995). The $hycE$ gene encodes the nickel-containing large subunit. A hydrogenase 3 small subunit analogue has not been identified. From the other $hyc$ gene products, only HycA and HycI have been characterized. HycA has a role in transcriptional regulation. HycI is a protease involved in post-translational procession of HycE.

Apart from the structural genes described above, a set of seven genes has been identified which are involved in the synthesis and insertion of the [NiFe] metal center and maturation of the enzymes (DRAPAL and BÖCK 1998). These are the products of the six so-called hyp genes (HypA, HypB, HypC, HypD, HypE, and HypF). In addition, maturation of hydrogenase enzymes require another gene encoding a specific endopeptidase ($hyaD$ for hydrogenase 1, $hybD$ for hydrogenase 2, or $hycI$ for hydrogenase 3) (MENON et al. 1991; ROSSMANN et al. 1995; FRITSCHE et al. 1999).

### 2.2.2 Virulence and [NiFe] hydrogenase 2

In *E. coli*, [NiFe] hydrogenase 2 is involved in the oxidation of anaerobic hydrogen by catalyzing the interconversion of molecular hydrogen to protons and electrons, and in the reduction of fumarate to succinate by contributing electrons from hydrogen oxidation to fumarate reductase (BALLANTINE and BOXER 1985; MENON et al. 1990). Additionally, this enzyme is transported by the membrane targeting and translocation (MTT; WEINER et al. 1998) or twine-argine translocation pathway (TAT; SARGENT et al. 1998) by which the correctly folded and full active dimer can cross the membrane (WU et al. 2000; BERKS et al. 2000). In order to be transported by the TAT system, the small subunit of [NiFe] hydrogenase 2 requires a signal peptide. This signal peptide contains a conserved (S/T)RRxFLK motif (BERKS 1996). The TAT system is present in a large number of microorganisms (DILKS et al. 2003). TAT-secreted proteins have been examined and they are mostly periplasmic enzymes taking part in complex, multiprotein oxidation-reduction systems involved in respiration or anaerobic growth (BERKS 1996; ROBINSON and BOLHUIS 2001). In *Pseudomonas aeruginosa*, the TAT system is required for the correct localization of proteins
involved in iron siderophore biosynthesis and uptake (OCHSNER et al. 2002). The TAT system also catalyses the first step in the export of phospholipases (VOULHOUX et al. 2001). These traits have been associated with virulence. Since these phospholipases are involved in the breakdown of eukaryotic membranes, and since iron acquisition usually is a pathogenicity factor in microorganisms, it is not surprising that Tat mutant strains of *Pseudomonas aeruginosa* have been found to be attenuated in virulence in an animal model (OCHSNER et al. 2002).

### 2.3 Bacterial fucose metabolism

#### 2.3.1 Organization and structural genes

L-Fucose is a naturally occurring methyl pentose that is metabolized in *E. coli* via an inducible pathway. This dissimilatory pathway is mediated sequentially by L-fucose permease (HACKING and LIN. 1976), L-fucose isomerase (GREEN and COHEN 1956), L-fuculose kinase (HEATH et al. 1962), and L-fuculose 1-phosphate aldolase (GHALMBOR and HEATH 1962) (Fig. 1). The pathway branches after the aldolase cleaves the intermediate, fuculose 1-phosphate, into dihydroxyacetone phosphate and L-lactaldehyde. Under aerobic conditions, the aldehyde is oxidized irreversibly to L-lactate by a NAD-linked dehydrogenase. This dehydrogenase is encoded by *ald*, which is not linked to the *fuc* gene cluster. L-lactate then enters the general metabolic pool by being converted to pyruvate (SRIDHARA and WU 1969; COCKS et al. 1974). Under anaerobic conditions, the aldehyde is reduced to L-1,2-propanediol by a NADH-linked oxidoreductase. The propanediol is then excreted into the medium (COCKS et al. 1974; LIN 1987).

The L-fucose structural genes of *E. coli* are organized as a regulon of at least two operons: the *fucPIK* operon encodes the permease, isomerase, and kinase, respectively; the *fucAO* operon encodes the aldolase and oxidoreductase, respectively (CHEN et al. 1989). An activator protein for the pathway, active in the presence of L-fuculose 1-phosphate, is encoded by *fucR* (CHEN et al. 1987).
2.3.2 **Fucose and bacterial virulence**

The *E. coli* L-fucose regulon appears to be under positive regulational control (CHEN et al. 1987) with L-fuculose 1-phosphate as apparent inducer (BARTKUS and MORTLOCK 1986; LIN 1987). Anaerobic metabolism of fucose requires the activity of propanediol oxidoreductase to reduce the L-lactaldehyde to L-1,2-propanediol, a fermentation product, using NADH as cofactor (COCK et al. 1974). JEFCOAT et al. (2001) showed that fucose levels are increased in the mucins of horses suffering from chronic inflammation of the airways. In addition, *Pseudomonas aeruginosa* produces fucose-binding lectin (PA-IIL) (GILBOA-GARBER 1982; GARBER et al. 1987). The lectin (PA-IIL) is associated with bacterial virulence, biofilm formation (GILBA-GARBER 1996), and bacterial binding to the receptor (IMUNDO et al. 1995). ADAM et al. (1997) demonstrated that the lectin PA-IIL could stop the ciliary beating in human airways in vitro. This inhibitory effect can be abolished by preincubating PA-IIL with its specific sugar receptor, fucose.

As it has been described in the literature review, a main concern in the control of *A. pleuropneumoniae* infection is the occurrence of carrier animals occurring even after vaccination. The bacterium can persist in the pig's tonsils and in necrotic lung lesions. Under these conditions, the bacterium encounters anaerobic conditions. Therefore, it was hypothesized that an impairment of the anaerobic metabolism might reduce the bacterial fitness under anaerobic conditions and thus result in attenuation or reduced persistence in the host. Based on this hypothesis, isogenic mutants with deletions in genes expressed under anaerobic conditions were constructed, and their possible role in virulence was investigated in an aerosol infection model.
Fig. 1: Pathways for aerobic and anaerobic dissimilation of L-fucose by *E. coli*
Materials and methods

3 Materials and methods

3.1 Bacterial cultures

3.1.1 Bacterial strains

Bacterial strains used in this study are listed in Table 1.

3.1.2 Media and growth condition

*E. coli* strains were cultured in Luria-Bertani (LB) medium supplemented with the appropriate antibiotics (100 µg/ml ampicillin, 25 µg/ml); for the cultivation of *E. coli* β2155, 1mM diaminopimelic acid was added. Bacteria were incubated at 37 °C in an incubator (Memmert GmbH & Co. KG, Schwalbach) or in a shaking incubator (Incubator shaker Series 25, New Brunswick Scientific Co., Inc., Edison, NJ, USA).

*A. pleuropneumoniae* was cultured in PPLO medium supplemented with PPLO-supplement and 0.1 % Tween 80. For the selection of *A. pleuropneumoniae* transconjugants, 25 µg/ml kanamycin was added. The cultures were incubated at 37 °C in a 5 % CO₂ incubator (Heraeus CO₂-Auto-Zero, Hereaus Instruments GmbH Labortechnik, Hanau) or in a shaking incubator.

**Media**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB-Medium</td>
<td>10 g Bacto-Trypton, 5 g Yeast extract (Difco), 5 g NaCl, add distilled water to 1 liter</td>
</tr>
<tr>
<td>LB Agar</td>
<td>LB broth with 1.5 % agar</td>
</tr>
<tr>
<td>PPLO broth</td>
<td>21 g/l PPLO broth, add distilled water to 1 liter</td>
</tr>
<tr>
<td>PPLO agar</td>
<td>35 g/l PPLO agar, 2 g agar, add distilled water to 1 liter</td>
</tr>
</tbody>
</table>

Note: All media were sterilized by autoclaving. PPLO broth was sterilized by filtration.

**Antibiotic solutions and supplements**

- **Ampicillin**: stock solution 100 mg/ml in 70 % ethanol, with addition of concentrated HCl until substance is completely dissolved.
- **Chloramphenicol**: stock solution 25 mg/ml in 70 % ethanol
- **Kanamycin**: stock solution 50 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: 1 g/l L-gultamine, 26 g/l L-cysteine dichloride, 1 g/l L-cystine dichloride, 1g/l nicotanamide dinucleotide (NAD) in 10% D (+) glucose monohydrate.

Antibiotic stock solutions and diaminopimelic acid stock solution were sterilized by filtration and stored at -20 ºC.

3.2 Isolation of DNA

3.2.1 Isolation of plasmid DNA

Plasmids used in this study are listed in Table 2.

3.2.1.1 Minipreparation of plasmid DNA

This method was modified from BIRNBOIM and DOLLY 1979.

1. Inoculate a single colony into 3 ml LB broth containing the appropriate antibiotic; incubate at 37 ºC with shaking (200 rpm) overnight.

2. Resuspend pellet in 300 µl solution I by repeated pipetting. Add 300 µl solution II and mix gently until the solution becomes homogenous, clear and viscous. Add 300 µl solution III and mix; in this step viscosity is reduced and a large precipitate of chromosomal DNA and cell debris is formed.

3. Centrifuge at 13,000 rpm for 15 minute to eliminate cell debris and chromosomal DNA.

4. Transfer about 900 µl of clear supernatant into a new eppendorf tube.

5. Precipitate plasmid DNA by addition of 600 µl cold isopropanol, mix thoroughly and leave at room temperature for 10 minutes. Centrifuge at 13,000 rpm for 15 minutes, discard supernatant and dry the pellet.

6. Resuspend in 50 µl A. bidest.

7. Analyze 2.5 µl of DNA solution by endonuclease restriction and gel electrophoresis.
Reagents

Solution I:  50 mM Tris-HCl, pH 8, 10 mM EDTA, RNase 0.1 mg/ml
Solution II:  0.2 M NaOH, 1 % SDS
Solution III:  3.2 M potassium acetate pH 5.5

Table 1: List of bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> DH5αF'</td>
<td>$\Phi$80dlacΔ(lacZ)M15</td>
<td>RALEIGH et. al. 1989</td>
</tr>
<tr>
<td><em>E. coli</em> β2155</td>
<td>thrB1004 pro thi hsdS lacZΔM15 (F' lacZΔM15 lacIq traD36 proA $^+$ proB $^+$) Δdap::erm (erm')</td>
<td>DEHIO and MEYER 1997</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em> AP76</td>
<td><em>A. pleuropneumoniae</em> serotype 7 strain kindly provided by the Western College of Veterinary Medicine, Saskatoon, Canada</td>
<td>ANDERSON et. al. 1991</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em> AP76 Δhyb</td>
<td>Unmarked hybB-negative knockout mutant of <em>A. pleuropneumoniae</em> AP76</td>
<td>This study</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em> AP76 Δfuo</td>
<td>Unmarked fucO-negative knockout mutant of <em>A. pleuropneumoniae</em> AP76</td>
<td>This study</td>
</tr>
</tbody>
</table>

3.2.1.2 Preparation of plasmid DNA (Midi-Prep with JetStar® Kit)

1. Inoculate a single colony into 2.5 ml LB broth containing the appropriate antibiotic and incubate at 37 °C overnight.
2. Inoculate overnight culture in 25 ml LB broth containing appropriate antibiotic and incubate with shaking (200 rpm) until OD$_{660}$ = 0.8-0.9. Add chloramphenicol (100 µg/ml) and incubate overnight with shaking.
3. Bacteria were pelleted by centrifugation at 6000 rpm for 15 minute, 20 °C.
4. Column was equilibrated with 10 ml solution E4.
5. Pellet was resuspended with 4 ml solution E1 until the suspension was homogenous.
6. Add 4 ml solution E2 and mix gently until lysate appears to be homogeneous. Do not vortex.
7. Add 4 ml solution E3 and mix.
8. Column was loaded with cell suspension from step 7 through filter paper.
9. Wash the column twice with 10 ml solution E5.
10. Elute the DNA with 5 ml solution E6.
11. Add 3.5 ml cold isopropanol to precipitate DNA.
12. Centrifuge at 4 ºC and 8000 rpm for 20 minute.
13. Wash the DNA with 70 % cold Ethanol.
14. Centrifuge at 4 ºC and 8000 rpm for 20 minute.
15. Dry the pellet and resuspend in appropriate volume of A. bidest.

3.2.2 Isolation of chromosomal DNA from *A. pleuropneumoniae*

1. Bacterial cultures grown overnight on solid medium were harvested by resuspending the culture in 5 ml PPLO medium and transferred to a 10 ml polypropylene tube; bacterial cells were recovered by centrifugation (Sorvall RC-5B Refrigerated Superspeed Centrifuge, Du Pont Inst., Bad Hamburg, Germany) at 5,000 rpm (SA600 Rotor) at 4 ºC for 10 min, the supernatant was discarded.
2. Lysis of bacterial cells was achieved by addition of 5 ml lysis buffer with proteinase K (10mM EDTA pH 8.0, 0.1 % SDS, 0.5 mg/ml proteinase K) and mixed gently by inversion. The mixture was incubated at 55 ºC for 1h.
3. RNA contamination was removed by the addition of RNase to a final concentration 100 µg/ml and further incubation at 37 ºC for 20 min.
4. To remove proteins, ¼ volume of phenol equilibrated in TE pH 7.8 (Roti® Phenol, Roth, Karlsruhe) to the DNA solution was added and mixed. ¼ volume of chloroform isoamylalcohol (24:1) was added, mixed and centrifuged at 12,000 rpm for 10 min.
5. The top (aqueous) phase containing DNA was carefully removed by using a plastic pasteur pipette and transferred to a new tube.
6. Chloroform-isoamylalcohol extraction was repeated until no interphase was visible.
7. The upper phase was transferred into a new tube, and DNA was precipitated by adding 0.1 volume of 3 M Na-acetate (pH 5.2) and 1 volume of isopropanol.
8. The DNA thread generated by careful inversion of the tube was collected with a small pipette tip, washed in 70 % ethanol for 5 min and finally in 96 % ethanol for 5 min.
9. DNA was dissolved in 200 µl A. bidest. overnight at 4 ºC. 5 µl of DNA were analyzed by gel electrophoresis.
### Materials and methods

#### Source
- Stratagene
- OSWALD et al. 1999
- Amersham Bioscience
- BALTES 2002
- This study

#### Characteristics
- *E. coli* cloning vector carrying an ampicillin resistant determinant
- Transconjugation vector based on pBluescript SK with *mob*RP4, polycloning site, Tn903-derived kanamycin determinant, and transcriptional fusion of the *omlA* promotor with *sacB* gene
- *Escherichia coli* vector carrying an ampicillin resistance determinant, devised to construct GST fusion proteins
- pGH433 carrying the *hyb* gene on an bp insert in the *Bgl*II restriction site
- *NcoI* deletion of pRN400
- pBluescript SK carrying a *Hae*III-*Nsi*I fragment from pRN401
- *Hind*III-*Nar*I deletion of pHYB101 after fill-in of cohesive ends with Klenow fragment
- pBMK1 carrying a *ApaI-Not*I fragment from pHYB102
- pGEX5x3 carrying a *Hae*III fragment from pRN400
- pGH433 carrying an *fuc* operon at *Bgl*II restriction site

#### Table 2: Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Characteristics</th>
<th>Source (Reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript SK</td>
<td><em>E. coli</em> cloning vector carrying an ampicillin resistant determinant</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBMK1</td>
<td>Transconjugation vector based on pBluescript SK with <em>mob</em>RP4, polycloning site, Tn903-derived kanamycin determinant, and transcriptional fusion of the <em>omlA</em> promotor with <em>sacB</em> gene</td>
<td>OSWALD et al. 1999</td>
</tr>
<tr>
<td>pGEX5x3</td>
<td><em>Escherichia coli</em> vector carrying an ampicillin resistance determinant, devised to construct GST fusion proteins</td>
<td>Amersham Bioscience</td>
</tr>
<tr>
<td>pRN400</td>
<td>pGH433 carrying the <em>hyb</em> gene on an bp insert in the <em>Bgl</em>II restriction site</td>
<td>BALTES 2002</td>
</tr>
<tr>
<td>pRN401</td>
<td><em>NcoI</em> deletion of pRN400</td>
<td>This study</td>
</tr>
<tr>
<td>pHYB101</td>
<td>pBluescript SK carrying a <em>Hae</em>III-<em>Nsi</em>I fragment from pRN401</td>
<td>This study</td>
</tr>
<tr>
<td>pHYB102</td>
<td><em>Hind</em>III-<em>Nar</em>I deletion of pHYB101 after fill-in of cohesive ends with Klenow fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pHYB603</td>
<td>pBMK1 carrying a <em>ApaI-Not</em>I fragment from pHYB102</td>
<td>This study</td>
</tr>
<tr>
<td>pHYB504</td>
<td>pGEX5x3 carrying a <em>Hae</em>III fragment from pRN400</td>
<td>This study</td>
</tr>
<tr>
<td>pRN402</td>
<td>pGH433 carrying an <em>fuc</em> operon at <em>Bgl</em>II restriction site</td>
<td>BALTES 2002</td>
</tr>
</tbody>
</table>
**Materials and methods**

**Source**

REFERENCE

This study

**Characteristics**

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Characteristics</th>
<th>Source (Reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pFUO 103</td>
<td>Ligation of HindIII-ScaI fragment from pRN402 into pBlueskript SK HindIII-SmaI restriction site</td>
<td>This study</td>
</tr>
<tr>
<td>pFUO104</td>
<td>deletion of XhoI restriction site of pFUO103 in Vector</td>
<td>This study</td>
</tr>
<tr>
<td>pFUO105</td>
<td>XhoI-PacI deletion of pFUO104 after fill-in of cohesive ends with Klenow fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pFUO604</td>
<td>Ligation of ApaI-NotI fragment from pFUO105 into pBMK1 ApaI-NotI restriction site</td>
<td>This study</td>
</tr>
</tbody>
</table>
3.3 DNA purification

3.3.1 DNA purification from TAE-agarose gels by adsorption to a silica matrix.

This method was performed by using the Geneclean® II kit (Bio 101, Vista, California, USA)

1. Excise DNA band from the ethidium bromide-stained agarose gel with a razor blade sterilized by flaming; the DNA is visualized using long-wave UV light (356 nm) for as short a time as practical. Transfer excised agarose gel into a weighed eppendorf tube, weigh agarose and determine approximate volume of gel by weight (0.1 g equals approximately 100 µl).

2. Add 3 volumes of NaI solution, incubate at 55 ºC in water bath until all agarose is completely melted.

3. Add 5 µl resuspended glassmilk for 5 µg DNA to the solution (additional 1 µl glassmilk for each 0.5 µg of DNA above 5 µg), mix by vortexing and incubate on ice for at least 30 min.

4. Pellet glassmilk binding the DNA by centrifugation at 13,000 rpm for 30 sec.

5. Wash pellet three times with 500 µl ice-cold NEW Wash solution and recentrifuge as in step 4; after the third wash, the pellet is additionally centrifuged at 13,000 rpm for 2 min. to ensure that residual supernatant can be removed.

6. Elute DNA from glassmilk by resuspending pellet in 25 µl A. bidest. and incubating in a 55 ºC water bath for 3 min.

7. Glassmilk is pelleted by centrifugation at 13,000 rpm for 2 min.

8. Carefully collect 20 µl of supernatant containing DNA into a new tube.

9. The 5 µl remaining supernatant is used to determine the concentration of DNA by gel electrophoresis and comparison with λ DNA digested HindIII. In the case of DNA purification for ligation, vector and insert DNA concentrations are compared in the same gel.
3.4 **Plasmid construction**

3.4.1 **Digestion of DNA with restriction endonucleases**

For the analysis of plasmid DNA, 0.5 to 1 µg DNA was used in a 25 µl reaction volume. In case of a preparative plasmid or chromosomal DNA digestion, 2 to 5 µg DNA were used in a 50 µl reaction volume. The concentration of DNA in the reaction should not exceed 0.05 to 0.1 µg/µl. The volume of restriction endonuclease should not exceed 10 % of the total reaction volume.

1. Mix the DNA solution with an appropriate reaction buffer and bovine serum albumin (BSA) if required according to the manufacture’s instruction, and Aq. bidest. to obtain a total volume as described above.
2. Add 5 to 10 units enzyme into the mixture.
3. In the case of plasmid DNA digestion, the reaction was incubated for 2 h at the appropriate temperature as recommended by the manufacturer. In case of chromosomal DNA digestion, the reaction was incubated overnight.
4. Analyze the digested DNA by agarose gel electrophoresis.

3.4.2 **Agarose gel electrophoresis**

1. Dissolve agarose powder in an appropriate buffer by boiling in a microwave oven (0.5 x TBE buffer for analytical gels or 1 x TAE buffer for preparative gels). The concentration of agarose in the gels depends on the size of the DNA fragments to be separated.
2. Cool the gel to about 55 °C and add the ethidium bromide solution (10 mg/ml) to a final concentration of 0.2 µg/ml (1µl : 50 ml agarose gel) and pour into the gel tray.
3. Mix the DNA sample with a loading buffer and load into the gel slot. Standard DNA marker (HindIII-digested bacteriophage λ DNA, 1 kb DNA ladder or 100 bp DNA ladder, New England Biolab, Schwalbach, Taunus) was run along with the sample. Set the voltage to 6 V/cm of the electrode-distance and run the gel until the bromophenol blue dye in the loading buffer has migrated a distance sufficient for the separation of the DNA fragments.
4. The gel was visualized on a UV transilluminator and photographed with a camera (Polaroid) or an electronic image documentation system (Gel Doc 1000/Multi-analyst, Bio-Rad).

**Reagent**

3.4.3 **Generation of blunt-end DNA by 5' overhang fill-in**

1. Add each dNTP to a final concentration at 33 µM into completely digested DNA in restriction endonuclease reaction.

2. Add 1 unit of Klenow fragment (NEW England Biolabs, Schwach, Taunus) per µg of DNA and incubate 15 min at 25 ºC.

**Reagent**

dNTP stock solution: 10 mM dATP, 10 mM dCTP, 10 mM dTTP, 10mM dGTP

3.4.4 **Generation of blunt-end DNA by 3' overhang removal**

1. Add 1 to 3 units of T4 DNA polymerase (NEW England Biolabs, Schwach, Taunus) into completely digested DNA in a restriction endonuclease reaction.

2. Incubate at 16 ºC for 20 min.
3.4.5 Alkaline phosphatase treatment

In order to prevent self-ligation of vector DNA without insert DNA, the calf intestinal phosphatase (CIP) is used for dephosphorylation of the 5' ends of the vector.

1. Add 0.5 µl (5 units) of CIP (NEW England Biolabs, Schwach, Taunus) to completely digested DNA in a restriction reaction.
2. Incubate at 37 ºC for 60 min.

3.4.6 Ligation

1. Vector DNA and insert DNA fragments are separated by agarose gel electrophoresis, and purified from agarose gel by using Geneclean II kit.
2. Mix vector DNA and insert DNA, 2 µl 10 x ligation buffer and Aq. bidest. to make a 20µl total volume. The molar ratio of vector to insert was set to approximately 1:2 and 1:5, and 0.5 µl ligase was added.

Two controls were included as follows.
   a) vector only  b) vector and ligase
3. The ligation reactions were incubated at 16 ºC overnight.

3.5 Transformation

3.5.1 Preparation of E. coli chemical competent cells for transformation

1. Inoculate a single colony into 1.5 ml LB broth containing 20 mM MgCl₂, incubate at 37 ºC. Prepare 150 ml LB broth containing 20 mM MgCl₂ in a 1-litre flask and incubate at the same conditions to test sterility of medium.
2. Inoculate 1.5 ml overnight culture in prewarmed and sterility-tested LB broth from step1. Grow at 37 ºC with shaking (200 rpm) to OD₆₀₀ of 0.3 to 0.4.
3. Place the flask on ice for 30 min. All following steps are processed on ice.
4. Harvest bacterial cells by centrifugation at 4 ºC, 5,000 rpm for 10 min., discard supernatant.
5. Resuspend cell pellet in 30 ml cold TFB1 solution and place the mixture on ice for at least 90 min.
6. Centrifuge at 4 °C, discard supernatant, resuspend the pellet in 5 ml TFB2 solution.
7. Aliquot 300 µl of competent cells into prechilled sterile microcentrifuge tubes. Competent cells can be used immediately or stored at −70 °C.
8. Test quality of competent cells by transformation.

**Reagent**

TFB1: 0.29 g potassium acetate (30 mM), 1.21 g RbCl (100 mM), 0.11 g CaCl₂ (10 mM), 0.99 g MnCl₂ (50 mM), 50 % glycerine 30 ml, add A. bidest. to 100 ml, adjust pH to 5.8 with 0.2 M acetic acid

TFB2: 0.21 g MOPS (10 mM), 0.83 g CaCl₂ (75 mM), 0.12 g RbCl (10 mM), 50 % glycerine 30 ml, add A. bidest to 100 ml, adjust pH to 6.5 with 1 M KOH

These solutions were sterilized by filtration and stored at 4 ºC.

### 3.5.2 Transformation of *E. coli* by heat shock

1. Thaw the frozen competent cells on ice.
2. Distribute 100 µl competent cells in microcentrifuge tubes; add 0.5 µg DNA or half the volume of a ligation reaction. Two controls should be included. One sample should receive a circularized plasmid known to be capable of transforming the strain, and another should not contain DNA. Incubate on ice for 30 min.
3. Heat shock was applied at 42 ºC for 5 min and then chilled on ice for 2 min.
4. Add 400 µl prewarmed LB broth and incubate at 37 ºC for 1h. Plate onto an agar containing the appropriate selective antibiotic and onto LB agar without antibiotic to check viability of competent cells.
5. Incubate at 37 ºC overnight.
3.6 Transconjugation from *E. coli* to *A. pleuropneumoniae* by filter mating technique

In this study, the mutant gene cloned into the mutagenesis vector (pBMK1) was mobilized from *E. coli* B2155 (ΔdapA), a diaminominopimelic acid auxotrophic donor strain, into the *A. pleuropneumoniae* recipient.

1. Grow donor and recipient on appropriate solid medium and incubate overnight.
2. Remove culture with a sterile cotton swab and resuspend in TNM buffer (separate tubes, do not vortex), determine the cell density at OD$_{600}$.
3. Place nitrocellulose disc (0.45 µM pore size, 2.5 cm diameter, Millipore, Eschborn) onto sterile gel blotting paper in a petri dish.
4. Aliquots corresponding to 0.1 ml donor and 0.8 ml of recipient (each at OD$_{600} = 1$) are mixed by carefully repeated pipetting and transferred onto the nitrocellulose disc (from step 3). Allow the buffer to be absorbed briefly.
5. A control reaction was included by mixing 0.1 ml donor with 0.8 ml of TNM buffer.
6. Transfer the discs onto the prewarmed PPLO agar containing 1 % IVX, 1 mM diaminopimelic acid and 10 mM MgSO$_4$, and incubate at 37 ºC in CO$_2$ incubator for 7 hours.
7. Place the filter in a microcentrifuge tube containing 650 µl PPLO broth, wash out bacteria from the filter by vortexing.
8. Plate the cell suspension onto PPLO agar supplemented with IVX and 25 µg/ml kanamycin, incubate at 37 ºC in a 5 % CO$_2$ incubator overnight.

**Reagent**

TNM buffer: 1 mM Tris-HCl pH 7.2, 100 mM NaCl, 10 mM MgSO$_4$

3.7 Sucrose counterselection

1. A single colony of *A. pleuropneumoniae* confirmed to carry the kanamycin resistance determinant on the chromosome resulting from a plasmid cointegrate, was inoculated into 1 ml of supplemented PPLO broth that had been prewarmed at 37 ºC, 5 % CO$_2$ overnight.
2. Incubate at 37 ºC with shaking (200 rpm) for 2 h or until the culture is slightly turbid.
3. Add 400 µl 2.5 x supplemented PPLO (salt free), 500 µl sucrose (40 %), and 100 µl sterile equine serum.
4. Incubate at 37 ºC with shaking (200 rpm) for 6 h.
5. Plate aliquots of 50 µl on supplemented PPLO agar without kanamycin and incubate overnight at 37 ºC, 5% CO₂.

Preparation of 2.5 x salt free PPLO broth

1. 46 g Bacto Beef Heart For Infusion (Difco) in 1 l A. dest. was kept at 50 ºC with constant stirring for 1 h, boiled in microwave oven for 3 min. and cooled to room temperature with constant stirring.
2. Filtrate the suspension to remove solids, add 7.4 g Bacto peptone (Difco).
3. Adjust pH to 7.4 with 1 M NaOH and sterilize by filtration.

3.8 Polymerase chain reaction (PCR)

PCR was performed in a thermocycler (Cyclone Thermocycler, Integra, Biosciences, MA, USA or Crocodile III, Appligene, Illkirch, France) in a 25 µl total reaction volume using Taq DNA polymerase (GIBCO BRL, Eggenstein). The mixtures were prepared on ice by addition of the reagents in the order described in the Table 3. Primers used in this study are listed in Table 4. The reaction premixes were prepared, and 20 µl were aliquoted for each reaction, subsequently 5 µl template DNA (about 10 ng) was added and finally 30 µl mineral oil was overlaid for reactions in the 'Cyclone' cycler. In the case of screening deletion mutants following sucrose counterselection, the DNA template was prepared by boiling a single colony. Before the beginning of first cycle, the template was completely denatured at 94 ºC for 3 min, subsequently PCR amplification was performed for 32 cycles. Each cycle was carried out with the following steps; denaturing at 94 ºC for 30 sec, annealing at 54 ºC for 40 sec, and primer extension at 72 ºC for 1 min for every 1 kb length of DNA fragment to be amplified. After the last cycle, the final extension was performed for 10 min at 72 ºC. Finally, PCR products were analyzed by gel electrophoresis.
Isolation of DNA template by colony boiling

1. Pick a single colony with a small pipette tip and resuspend in 100 µl TE buffer (1:10 diluted) in a microtiter plate or eppendorf tube.
2. Boil in microwave at 180 watts for 8 min.
3. Use 5 µl for a PCR in a 25 µl total reaction volume.

Reagent

TE buffer: 10 mM Tris HCl pH 8.0, 0.1 mM EDTA pH 8.0

Table 3: Component in the PCR reaction

<table>
<thead>
<tr>
<th>Reaction component</th>
<th>Stock solution</th>
<th>Final concentration</th>
<th>Volume reaction (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. bidest</td>
<td>-</td>
<td>-</td>
<td>11</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>50 mM</td>
<td>1.5 mM</td>
<td>0.75</td>
</tr>
<tr>
<td>dNTPs</td>
<td>10 mM</td>
<td>0.2 mM</td>
<td>0.5</td>
</tr>
<tr>
<td>PCR buffer</td>
<td>10x</td>
<td>1x</td>
<td>2.5</td>
</tr>
<tr>
<td>Forward primer</td>
<td>5 pmol/µl</td>
<td>0.5 pmol/µl</td>
<td>2.5</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5 pmol/µl</td>
<td>0.5 pmol/µl</td>
<td>2.5</td>
</tr>
<tr>
<td>Taq DANN polymerase</td>
<td>5 U/µl</td>
<td>1.25 U</td>
<td>0.25</td>
</tr>
<tr>
<td>Template</td>
<td>-</td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>Final volume</td>
<td>-</td>
<td>-</td>
<td>25</td>
</tr>
</tbody>
</table>
### Table 4: Primers used in this study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Characteristics</th>
<th>Source (Reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN34-FW RN34-RW</td>
<td>5' TTA GCC AGA GTA GCG GCA AT 3'&lt;br&gt;5' CGG AAA ATT TGT CGC CTA AG 3'&lt;br&gt;internal primers pair of RDA fragment RN34</td>
<td>BALTES 2002</td>
</tr>
<tr>
<td>o34-1f</td>
<td>5' GCC AGC TTA TTC GGA TAT ACC 3'&lt;br&gt;upstream primer comprising position 290-310 of the <em>hyb</em> homologue</td>
<td>This study</td>
</tr>
<tr>
<td>o34-1r</td>
<td>5' AAT AGC GTG TAC CGT CGT ACA 3'&lt;br&gt;downstream primer comprising position 1399-1419 of the <em>hyb</em> homologue</td>
<td>This study</td>
</tr>
<tr>
<td>o34-2f</td>
<td>5' CTC CGA AAC GAA CGA CGA TAA 3'&lt;br&gt;upstream primer comprising position 793-803 of the <em>hyb</em> homologue</td>
<td>This study</td>
</tr>
<tr>
<td>o34-2r</td>
<td>5' TAT CGT CGT TCG TTT CGG AGA 3'&lt;br&gt;downstream primer comprising position 794-804 of the <em>hyb</em> homologue</td>
<td>This study</td>
</tr>
<tr>
<td>RN37-FW RN37-RW</td>
<td>5' GCT TCC GAC GTT ATC CAA AG 3'&lt;br&gt;5' TCA GGA TAA ATG GCA CCA CA 3'&lt;br&gt;internal primer pair of RDA fragment RN37</td>
<td>BALTES 2002</td>
</tr>
<tr>
<td>oRN37-1f</td>
<td>5' TTC CCT GAC CGG GTG ATT TTT 3'&lt;br&gt;downstream primer comprising position 5815-5835 of the <em>fuc</em> operon homologue</td>
<td>This study</td>
</tr>
<tr>
<td>oRN37-1r</td>
<td>5' CGT TGA TTG GTG GTA AGA CTG 3'&lt;br&gt;upstream primer comprising position 925-945 of the <em>fuc</em> operon homologue</td>
<td>This study</td>
</tr>
</tbody>
</table>
### Table 4 continued

<table>
<thead>
<tr>
<th>Primers</th>
<th>Characteristics</th>
<th>Source (Reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oRN37-2f</td>
<td>5' CGA CAT CTA TCG CAA GCT GTT 3' downstream primer comprising position 5644-5666 of the <em>fic</em> operon homologue</td>
<td>This study</td>
</tr>
<tr>
<td>oRN37-2r</td>
<td>5' TCG GCA TAT CTC GTC GAA ACT 3' upstream primer comprising position 1877-1897 of the <em>fic</em> operon homologue</td>
<td>This study</td>
</tr>
<tr>
<td>oRN37-4f</td>
<td>5' GGC TGC AAT GAT CGG AAT ACA 3' downstream primer comprising position 5052-5072 of the <em>fic</em> operon homologue</td>
<td>This study</td>
</tr>
<tr>
<td>oRN37-4r</td>
<td>5' CGA ACA AAT GAC CGA AGA CAG 3' upstream primer comprising position 2693-2703 of the <em>fic</em> operon homologue</td>
<td>This study</td>
</tr>
<tr>
<td>oRN37-5f</td>
<td>5' CGC CCA TAC CGT CAA TAA ACA 3' downstream primer comprising position 4237-4257 of the <em>fic</em> operon homologue</td>
<td>This study</td>
</tr>
<tr>
<td>oRN37-5r</td>
<td>5' TCT CGT GCA GTT CGC ATT CTA 3' upstream primer comprising position 3411-3431 of the <em>fic</em> operon homologue</td>
<td>This study</td>
</tr>
<tr>
<td>oRN37-6f</td>
<td>5' CGC ATC ACG TTT TTC CGA TTC 3' downstream primer comprising position 3754-3774 of the <em>fic</em> operon homologue</td>
<td>This study</td>
</tr>
<tr>
<td>oRN37-6r</td>
<td>5' GTT TAA CTA AAG CGG AAG CGC 3' upstream primer comprising position 4082-4102 of the <em>fic</em> operon homologue</td>
<td>This study</td>
</tr>
<tr>
<td>oRN37-7r</td>
<td>5' TTG TCG CTA TCG CTA TCT ACG 3' upstream primer comprising position 4489-4509 of the <em>fic</em> operon homologue</td>
<td>This study</td>
</tr>
<tr>
<td>Primers</td>
<td>Characteristics</td>
<td>Source (Reference)</td>
</tr>
<tr>
<td>------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>oRN37-8f</td>
<td>5' GAG ACG TGT CAT TTC AAG GCA 3' downstream primer comprising position 2587-2607 of the fuc operon homologue</td>
<td>This study</td>
</tr>
<tr>
<td>oRN37-8r</td>
<td>5' GGG AAT TAA CCG ATG CGT TAC 3' upstream primer comprising position 5290-5310 of the fuc operon homologue</td>
<td>This study</td>
</tr>
<tr>
<td>oRN37-9f</td>
<td>5' TTT CGA CGA GAT ATG CCG ACA 3' downstream primer comprising position 1875-1895 of the fuc operon homologue</td>
<td>This study</td>
</tr>
<tr>
<td>oRN37-9r</td>
<td>5' GGC GAA TAA CTT GAC CGG TTT 3' upstream primer comprising position 5290-5310 of the fuc operon homologue</td>
<td>This study</td>
</tr>
<tr>
<td>oRN37-10r</td>
<td>5' GCG GAA TGG CCT TTA TAC GAA 3' upstream primer comprising position 6513-6533 of the fuc operon homologue</td>
<td>This study</td>
</tr>
<tr>
<td>oFUO-1f</td>
<td>5' AAG CAT CCG GTC GCA ATT ATC 3' upstream primer comprising position 4911-4931 of the fuc operon homologue</td>
<td>This study</td>
</tr>
<tr>
<td>oFUO-1r</td>
<td>5' GGC TCG GAT TTG TTT GTT CGA 3 downstream primer comprising position 6026-6046 of the fuc operon homologue</td>
<td>This study</td>
</tr>
<tr>
<td>oFUO-2f</td>
<td>5' AGA AAG CGT TAG TGG TTA CCG 3' upstream primer comprising position 4753-4773 of the fuc operon homologue</td>
<td>This study</td>
</tr>
<tr>
<td>oFUO-2r</td>
<td>5' AAT TCG ACC GCT TGC ACC AAA 3' downstream primer comprising position 6682-6702 of the fuc operon homologue</td>
<td>This study</td>
</tr>
</tbody>
</table>
3.9 Southern blot

3.9.1 Southern blotting

1. Digest the DNA sample to study with appropriate restriction endonuclase and separate DNA by gel electrophoresis. Include in the agarose gel a size standard such as λ DNA cut with HindIII. Photograph the gel with a ruler on a UV transilluminator.

2. Partially depurinate the DNA by transferring the gel in a plastic box by adding a 10 fold volume of 0.25 M HCl. Gently rock the gel in the solution on a moving platform for 15 min, repeat once. Rinse the gel with Aq. bidest. 2 times.

3. Denature the DNA by gentle rocking of the gel in the solution (1.5 M NaCl, 0.5 NaOH) for 1 h on a moving platform.

4. Neutralize the DNA by gentle rocking of the gel in the solution (1.5 M NaCl, 1 M Tris-HCl pH 8.0) for 1 hour on a moving platform.

5. Set up the gel for blotting by the capillary method (following from SOUTHERN 1975) onto nylon membrane (Positive membrane, Appligene, Illkirch, France) in 10 x SSC transfer buffer overnight.

6. Mark all slots on the membrane with a pencil and cut a corner of the blot to identify the orientation of the blot.

7. Wash the membrane with 5 x SSC for 5 min.

8. Dry the membrane on a filter paper in the air.

9. Bake the membrane in oven (Booskamp, Wuppertal) at 80 °C for 30 min. to fix the DNA on the membrane, keep the membrane in sealed plastic bag at room temperature.

Reagent

Depurination solution: 0.25 M HCl
Denaturation solution: 1.5 M NaCl, 0.5 M NaOH
Neutralization solution: 1.5 M NaCl, 1 M Tris-HCl pH 8.0
20 x SSC stock solution: 3 M NaCl, 0.3 M tri-sodiumcitrate-dihydrate
3.9.2 Labelling of probe with $\alpha^{32}$P-dCTP using the random priming method

1. 20 to 30 ng DNA are diluted in A. bidest in a 15.5 µl total volume, denatured at 100 °C for 5 min and placed on ice briefly.
2. Add 5 µl OLB solution, 1 µl of 10 mg/ml acetylated BSA, 1 µl Klenow fragment and 2.5 µl of 10µCi/µl $\alpha^{32}$P-dCTP.
3. Incubate at room temperature for 4 h.
4. Add 100 µl stop buffer. The labeled DNA probe can be used immediately or the unincorporated $\alpha^{32}$P-dCTP can be eliminated using a Sephadex G-25 column (NAP™-5 column, Pharmacia Biotec, Freiburg). The probe is stored in a lead container at -20 °C.

Reagent

OLB solution: 100 µl solution A, 250 µl solution B, 150 µl solution C stored at –20 °C
Solution A: 1 ml solution O (625 µl 2 M Tris-HCl pH 8.0, 125 µl 1 M MgCl, 250 µl A. bidest.), 18 µl mercaptoethanol, 5 µl 0.1 M dTTP, 5 µl 0.1 M dATP, 5 µl 0.1 M dGTP
Solution B: 2 M HEPES buffer pH 6.6 (adjust pH with NaOH)
Solution C: Hexadeoxyribonucleotide in TE buffer (pd(N)6, Pharmacia Biotec)
Stop buffer: 20mM NaCl, 20 mM Tris-HCl pH 7.5, 2 mM EDTA pH 8.0, 1 µM dCTP
TE buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0

3.9.3 Southern blot hybridization

1. Roll the membrane and pack in a hybridization tube, soak the membrane with 6 x SSC.
2. Prehybridize the membrane in 20 ml prehybridization solution in a hybridization oven (Minihybridization oven, Appligene) at 65 °C for 2 h.
3. Labeled DNA probe was denatured at 95 °C for 5 min, cooled on ice and then added to a membrane in 10 ml hybridization solution. Blots were hybridized at 55 °C overnight.
4. Wash the blot to remove the unspecifically bound probe in wash solution (wash solution and temperature depend on the stringency desired; in most applications, wash solution II were used for 2 h).

5. Place blot onto the filter paper to remove excess fluid.

6. Wrap the blot in plastic wrap and expose to X-ray film (Kodak X-OMAT AR, SIGMA, Deisenhofen) with intensifying screen at -70 °C. The exposure time was adjusted depending on the signal strength.

Note: The hybridization oven and all of the solutions used in hybridization and wash process must be prewarmed at 55 ºC before use.

Reagent
Prehybridization buffer: 6 x SSC, 0.5 % SDS, 5 x Denhardt’s solution
Hybridization buffer: 6 x SSC, 0.5 % SDS, 5 x Denhardt’s solution, 0.01 M EDTA
Wash solution I: 3 x SSC, 0.5 % SDS at 55 ºC for low stringency
Wash solution II: 1 x SSC, 0.5 % SDS at 60 ºC for intermediate stringency
Wash solution III: 0.1 x SSC, 0.5 % SDS at 68 ºC for high stringency
50 x Denhardt’s solution: 1 % (w/v) polyvinylpyrrolidone, 1 % (w/v) Ficoll 40, 1% (w/v) BSA in A. bidest.

3.10 Colony blot

3.10.1 Colony blotting

1. Place a circular nitrocellulose membrane (Protran BA 85, Schleicher & Schuell, Dassel) onto an agar plate containing bacterial colonies to be tested including positive and negative control cultures. Use forceps that are flamed to surface sterilize them to handle the nitrocellulose membrane. Mark the membrane in three asymmetric locations by stabbing into the agar beneath with a needle. Mark the master petri dish at the same locations and keep the master plate at 4 ºC until the results of the screening procedure become available.

2. Place the blot (colony side up) onto plastic wrap with a puddle of 1 ml 0.5 NaOH, leave for 3 min.
3. Place the blot on a dry filter paper until the excess liquid on the blot is adsorbed (but do not dry the membrane).
4. Repeat as in step 2 using fresh 0.5 M NaOH, repeat step 3.
5. Transfer the blot on to plastic wrap with a puddle of 1 M Tris-HCl pH 7.4, leave for 5 min., blot the filter on a dry filter paper (as in step 3) and repeat.
6. Transfer the blot onto plastic wrap with a puddle of 1.5 M NaCl, 0.5 M Tris-HCl pH 7.4, leave for 5 min., blot the filter on a dry filter paper (as in step 3).
7. Let the blot be soaked in 5 x SSC for 20 sec.
8. Repeat step 7.
9. Allow the blot to dry at room temperature for 30-60 min.
10. Place the blot between two sheets of gel blotting paper, bake at 80 ºC for 2 h.

3.10.2 Colony blot hybridization

1. Let the baked membrane soak in A. bidest..
2. Prehybridize the blot in prehybridization buffer for 2 h at 55 ºC.
3. Gently rub the cellular debris of the blot with a gloved hand under running tap water.
4. Hybridize the blot in hybridization buffer at 55 ºC overnight.
5. Wash the blot as described in Southern blot hybridization (3.9.3).
6. Place the blot onto filter paper briefly to remove excess fluid.
7. Expose the blot to X-ray film as described in Southern blot hybridization (3.9.3).

3.11 Pulsed-field gel electrophoresis (PFGE)

3.11.1 Preparation of total DNA of *A. pleuropneumoniae*-embedded agarose plugs for pulsed-field gel electrophoresis

1. 1st day
   1.1 Plate *A. pleuropneumoniae* from a glycerine stock on supplemented PPLO agar and incubate in 5 % CO₂ overnight.
2. 2\textsuperscript{nd} day

2.1 Inoculate a single colony into 3 ml PPLO-IVX-0.1% Tween\textsuperscript{®} 80 broth and incubate in 5 % CO\textsubscript{2} incubator overnight.

3. 3\textsuperscript{rd} day

3.1 Inoculate 3 ml overnight culture in 30 ml in PPLO-IVX-0.1% Tween\textsuperscript{®} 80 broth, incubate at 37 °C with shaking (200 rpm) until OD\textsubscript{600} = 0.3 and place the culture on ice for 20 min.

3.2 Dissolve 1.2 % agarose gel (chromosome grade, BioRad Inc., Hercules, Califonia) in Aq. bidest. in microwave, cool the gel to about 55 °C until step 3.6.

3.3 Five ml of culture was centrifuged at 5,000 rpm at 4 °C for 10 min, remove all of the supernatant.

3.4 Resuspend the cell pellet in 5 ml ice-cold PETT IV-buffer and recentrifuge as in step 3.2, remove all of the supernatant.

3.5 Resuspend the cell pellet in 0.5 ml PETT IV-buffer and incubate briefly at 55 °C in a water bath.

3.6 Add 0.5 ml agarose gel to bacterial suspension, mix by repeated pipetting using 1 ml pipette-tip and pour into 100 µl plug molds (Sample CHEF Disposable Plug Mold, Bio-Rad, München). Allow the agarose to solidify at 4 °C for 10-15 min.

3.7 Remove the tape from the bottom of the 10-well strip. Remove the plugs from the mold by using the tap at the end of the tape as a tool. Push the plugs out from the mold by inserting the narrow end of the tape into the mold and release 5 plugs into a new polypropylene tube containing 3 ml lysis buffer and incubate the tube horizontally at 37 °C for 2 h, discard buffer.

3.8 Add 3 ml ESP buffer and incubate at 55 °C overnight.

4. 4\textsuperscript{th} day

4.1 ESP buffer is discarded and the plugs are washed two times with 3 ml A. bidest. for 15 min. by tube-rolling at room temperature, A. bidest. is discarded.

4.2 To inactivate residual proteinase K, wash two times at room temperature with 2 ml TE-PMSF for 30 min., remove excess liquid.

4.3 Wash the plugs with 3 ml A. bidest. for 15 min, remove solution.

4.4 Add 3 ml TE buffer, incubate for 30 min and discard buffer.
4.5 Add 5 ml TE buffer and store at 4 ºC until plugs are used.

Reagent

PETT IV-buffer: 1 M NaCl, 10 mM Tris-HCl pH 8.0, 10 mM Na₂EDTA
Lysis buffer: 1 M NaCl, 10 mM Tris-HCl pH 8.0, 0.2 M Na₂EDTA, 0.5 % N-laurylsarcosine, 0.2 % deoxycholate, 2 µg/ml RNase and 1 mg/ml lysozyme (RNase and lysozyme were added just before use)
ESP buffer: 0.5 M Na₂EDTA, 1 % N-laurylsarcosine, 1 mg/ml proteinase K (proteinase K was added just before use)
TE buffer: 10 mM Tris-HCl pH 8.0, 1 mM Na₂EDTA
PMSF stock solution: 100 mM phenylmethylsulfonyl fluoride in isopropanol store at –20ºC
TE-PMSF: 1.5 mM phenylmethylsulfonyl fluoride in TE buffer (PMSF was added just before use)

3.11.2 Restriction of DNA embedded in agarose gel

1. ¼ gel plug was cut and used for each reaction. Equilibrate the gel plug in 3 volumes of an appropriate restriction endonuclease buffer (without BSA) as supplied by the manufacture for 1 h at room temperature.

2. Change the buffer and add 10 U of enzyme, incubate overnight at the appropriate temperature according to manufacturer’s instructions.

3.11.3 Pulsed-field gel electrophoresis

1. Dissolve 0.8 % agarose gel in 0.5 x TBE buffer, cool at 55 ºC and pour in a gel casting platform (Bio-Rad Inc., Hercules, California), allow the gel to become solid and remove the gel comb.

2. Place the digested plug gel into the slot using a scalpel and a hook formed from a Pasteur pipette. Bacteriophage lambda concatemers embedded in agarose gel (New England Biolabs, Schwalbach, Taunus) and bacteriophage lambda DNA digested with HindIII mixing in DNA loading buffer are used as standard markers. The slots are sealed by covering with agarose gel.
Materials and methods

3. Remove gel casting plate and place the gel into the gel electrophoresis box, cover with cold 0.5 x TBE buffer to a depth of 2-3 mm.

4. Set up the CHEF-DR III pulsed-field electrophoresis system (Bio-Rad, München) to 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.

5. DNA is stained with ethidium bromide 0.2 µg/ml for 15 min and destained in A. bidest for 15 min.

6. Document the gel on a UV transilluminator and photograph with Polaroid camera or image documentation system.

3.12 Nucleotide sequencing and sequence analysis

Nucleotide sequencing was done by SeqLab, Göttingen, Germany. Sequence analyses were performed using the HUSAR program package supplied by Deutsches Krebsforschungszentrum Heidelberg, Germany (dkfz).

3.13 Challenge experiment in the pig

The *A. pleuropneumoniae* [NiFe] hydrogenase-2 deficient mutant constructed in this study was examined in pigs 7-9 weeks of age [permit no. 009i-(neu)42502-98/45]. And the other mutant *A. pleuropneumoniae Δfuo* constructed in this study was examined in pigs 7-9 weeks of age [permit no. 509c-42502-01/488] Pigs were infected via aerosol, thereby mimicking the natural route of infection.

3.13.1 Challenge experiment timeline

<table>
<thead>
<tr>
<th>Day</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>-7</td>
<td>Arrival at the facility, blood samples taken for enzyme linked immunosorbent assay (ELISA)</td>
</tr>
<tr>
<td>0</td>
<td>Aerosol infection</td>
</tr>
<tr>
<td>7</td>
<td>Collection of bronchoalveolar lavage fluid (BALF), blood samples taken</td>
</tr>
<tr>
<td>14</td>
<td>Blood samples taken</td>
</tr>
<tr>
<td>21</td>
<td>Collection of BALF, blood samples taken, pigs subjected to post mortem examination following euthanasia</td>
</tr>
</tbody>
</table>
3.13.2 Origin and housing of the animals

Thirtytwo outbred pigs 7 to 9 weeks of age were purchased from an \textit{A. pleuropneumoniae}-free herd (no clinical symptoms, no serological response in the ApxII-ELISA (LEINER et al. 1999), randomly assigned to four groups, 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 (ETS123). Groups were housed in separate isolation units with controlled temperature and ventilation.

3.13.3 Aerosol infection chamber

Infections were carried out in an aerosol chamber built by Impfstoffwerk Dessau Tornau (Dessau, Germany) based on the descriptions of Jacobsen et al. (JACOBSEN et al. 1996). This chamber allows the simultaneous infection of four pigs (7-12 weeks of age). The top of the chamber consists of an acrylic window, which allows easy surveillance of the animals during aerosol exposure. The chamber has two air vents equipped with filters, one of which is connected to a compressor (KNF Neuberger, Freiburg, Germany) used to exchange the air in the chamber. All tubing is made from either autoclaveable silicone or Teflon\textsuperscript{®}. The bacterial suspension is aerosolized via a nozzle (Model no. 97058, Schlick Duesen, Untersiemau, Germany) operated by compressed air (Linde, Hannover, Germany).

3.13.4 Preparation of bacteria for aerosolization

For aerosol infection, the \textit{A. pleuropneumoniae} strains were grown with shaking in a 100 ml culture (inoculated with 10 ml of an overnight liquid culture) for approximately 3 hours at 37 °C to OD\textsubscript{660} of 0.4. The culture was placed on ice, diluted 1:300 in ice cold NaCl (150 mM), and kept on ice until use for a maximum of 2 hours. Immediately prior to aerosolization, bacteria were further diluted 1:100 in ice-cold NaCl (150 mM) resulting in a total living cell count per 13 ml dose (for four pigs) of approximately $1 \times 10^5$/ml; upon aerosolization, this dose corresponds to approximately $1 \times 10^2$ \textit{A. pleuropneumoniae} cells per liter of aerosol in the chamber, a dose which had been titrated for the \textit{A. pleuropneumoniae} strain AP76 to induce severe but not fatal disease (TEUTENBERG-RIEDEL et al., unpublished data).
3.13.5 Aerosol infection

Four pigs were put in the chamber at a time. The nozzle was set to "5", achieving a broad spray for even distribution of the aerosol across the chamber; the valve regulating the flow of the fluid was set to "75", and the challenge dose was aerosolized over the course of approximately 2 min at a pressure of 2 bar. Ten minutes after complete aerosolization of the challenge dose, the air in the chamber was exchanged two times over duration of 20 min using a compressor, and then the pigs were led back to their stable.

3.13.5.1 Surveillance of the animals during the experiment

Pigs were examined clinically at least once a day or as needed. Body temperatures were recorded in charts for each pig along with clinical symptoms like depression, dyspnoea or coughing.

3.13.6 Bronchoalveolar lavage fluid (BALF)^1

For the collection of BALF, pigs were anaesthetized by intramuscular application of azaperone (Stresnil, Janssen GmbH, Neuss, Germany) (2 mg/kg) followed by an intramuscular injection of ketamine (Ursostamin, Serumwerk Bernburg Ag, Bernburg) (15 mg/kg) and immobilized in a specially designed hammock. To obtain BALF, a flexible bronchoscope (Type XP20, Fa. Olympus, Hamburg) was introduced into the bronchus of the right posterior cranial lobe. The tip of the bronchoscope was pushed into 'wedge position' as to seal the bronchus. Twenty ml of isotonic NaCl (prewarmed to 30 °C) were injected and recovered by applying a suction force of 0.2 to 0.5 bar using an especially designed vacuum pump (Endoaspirator, Fa. Georg Paudrach, Hannover, Germany). This washing process was repeated five times, and an average of 90 ml of BALF were obtained. The BALF was kept on ice for up to 2 hours until the bacteriological examination was performed.

^1 Collection of bronchoalveolar lavage was performed by Dr. I. Hennig-Pauka, Clinic for Pigs, Small Ruminants Forensic Medicine and Ambulatory Service, Veterinary School Hannover
3.13.6.1 Bacteriological examination of BALF

One ml of BALF was centrifuged (5000 rpm, 10 min), and the pellet was resuspended in 60 μl of NaCl (150 mM). Twenty microliter were plated on supplemented PPLO agar as well as on Gassner and CSB agar, respectively. The degree of total bacterial colonization (growth on CSB agar), as well as colonization by enterobacteria (growth on Gassner agar) and by \textit{A. pleuropneumoniae}-like bacteria (minimal growth with distinct hemolysis on CSB, good growth on supplemented PPLO agar) was assessed. In addition, the total bacterial number as well as the number of \textit{A. pleuropneumoniae} was assessed by serial 10-fold dilutions of non-concentrated BALF and plating on CSB and supplemented PPLO-agar. Some individual \textit{A. pleuropneumoniae}-like colonies were subcultured on supplemented PPLO agar and confirmed PCR-analysis using specific primers.

3.13.7 Postmortem examination

Pigs were euthanized following BALF collection on day 21 post infection by intravenous injection of 10 ml of Eutha 77® (Pentobarbital, Essex Pharma, München, Germany) per pig.

3.13.7.1 Determination of lung lesion scores

In order to assess lung damage after experimental infection, HANNAN et al. (1982) developed a simple scheme of lung lesion mapping and evaluation; by dividing the whole lung into lobes, and assessing a total possible score of 5 for each lobe (resulting in a maximum score of 35 for the entire lung), individual lesions are be mapped on a simplified lung chart in which each lobe is subdivided into triangles. The number of 'affected' triangles is then counted, and the score for this lung lobe calculated as a fraction of five (HANNAN et al. 1982). The European Pharmacopoeia as the reference method in vaccine trials for \textit{A. pleuropneumoniae} has adapted this scheme.
3.13.7.2 Bacteriological examination of organ samples

The bacteriological examination included surface swabs of affected and unaffected lung tissue, tonsil, bronchial lymph node, and heart muscle on supplemented PPLO agar as well as on Gassner and Columbia sheep blood (CSB) agar. Plates were interpreted as described for BALF (3.12.6.1).

3.13.8 Enzyme Linked Immunosorbent Assay (ELISA)

The humoral immune response of pigs was determined in two different ELISAs. In order to assess antibody levels directed against the ApxIIA toxin, a standardized ELISA based on the recombinant *A. pleuropneumoniae* ApxIIA protein as solid phase antigen was employed (LEINER et al. 1999). In order to assess antibody levels directed against outer membrane components, an ELISA based on the detergent extract of an iron-restricted *A. pleuropneumoniae Appwt* culture (GOETHE et al. 2000) as solid phase antigen was used.

1. The detergent extract was diluted 1:50 in coating buffer; Polysorb® 96-microwell plates (Nunc, Roskilde, Denmark) were coated with 100 µl per well at 4 °C for 16 h without subsequent blocking.
2. Wash the plates three time with 1 x PBS-Tween solution before the addition of serum.
3. Sera were initially diluted 1:100 and further twofold in the plates in PBST. An internal positive control (pool of sera taken three weeks post infection from pigs infected with *A. pleuropneumoniae Appwt*) and negative control (pool of sera taken from pigs prior to infection) was used on each plate.
4. Incubate the plate at room temperature for 1 h. and wash the plate three times with 1 x PBS-Tween.
5. Add 100 µl for each well goat anti-pig peroxidase conjugate into serum dilutions and were incubated for 1 h at room temperature.
6. Wash the plate three times with 1 x PBS-Tween.
7. Add 100 µl of 2,2-azino-di[3-ethylbenzthiazoline sulfonate] (ABTS) containing 0.002 % H₂O₂ as substrate and incubate for 15 min.
8. Add 100 µl 50 % Methanol for each well to stop reaction. Then measure the optical density (OD). The test was considered valid when the optical density (OD) of the negative serum at a 1:100 dilution was lower than the OD of the positive serum at a 1:12,800 dilution. The titer given is the serum dilution with an OD higher than twice the OD of the negative control serum at a 1:100 dilution.

Reagents

Coating buffer: 2.83 g NaHCO₃, 1.59g Na₂CO₃ add A. dest to 1 litre
10 x PBS-Tween: 80 g NaCl (1.36 M), 3 g KH₂PO₄ (1.5 mM), 14.2 g Na₂HPO₄ (0.1 M), 2 g KCl (27 mM), Tween-20 5 ml, add A. dest to 1 l
Citrate phosphate buffer: 0.1 M citric acid, adjust pH to 4.25 with disodiumphosphate (for ABTS-substrate) solution

3.13.9 Statistics

Statistical graphics were created using Plot-It (Scientific Programming Enterprises, Haslett, MI, U.S.A) the non-parametric Wilcoxon test was performed using the WinStat® plug-in module (R. Fitch Software, Staufen, Germany) for Microsoft Excel®. Differences p < 0.05 in the Wilcoxon test were considered significant.
4 Results

4.1 The *A. pleuropneumoniae* [NiFe] hydrogenase 2 gene (*hybB*)

4.1.1 Sequence analysis

In an experiment that involved differential screening of an *A. pleuropneumoniae* cDNA library (BALTES 2002), one plasmid, designated as pRN34, contained a 281 bp fragment, RN34, which contained a partial open reading frame (ORF) that was 54% identical to the [NiFe] hydrogenase 2 protein (HybB) of *E. coli* (GenBank accession no. P37180). Using fragment RN34 as a radiolabeled probe to screen an *A. pleuropneumoniae* genomic library (BALTES 2002), one plasmid, pRN400, was found to contain a complete 1182 bp ORF encoding for a putative *hybB* gene of 393 amino acids in length. The predicted protein was 66% identical to the HybB protein of *E. coli*. In a BLAST search (ALTSCHUL et al. 1990) on the incomplete genomic sequence of *A. pleuropneumoniae* serotype 7 (GenBank accession no. NC_004427) with the *A. pleuropneumoniae* AP76 *hybB* ORF identified, putative *hybA*, *hybC*, *hybD*, *hybE*, *hybF*, and *hybG* ORFs were found to be located in the vicinity of the *hybB* ORF. These ORFs are arranged in one operon in the order *hybABCDEGF*, with the *hybF* ORF being arranged in the opposite direction to the others. The *hybA* ORF contains a recognition sequence for the twin arginine translocation (TAT) pathway (BERKS 1996). The nucleotide and amino acid sequences are listed in appendix 8.2.

4.1.2 Construction and characterization of an isogenic *A. pleuropneumoniae* [NiFe] hydrogenase 2 mutant

4.1.2.1 Construction of plasmid pHYB603 for the introduction of a [NiFe] hydrogenase 2 deletion into *A. pleuropneumoniae* AP76

In order to construct pHYB603 (Fig. 2) used for transconjugation, the *Hae*III-*Nsi*I fragment containing the 1182 bp [NiFe] hydrogenase gene from pRN401 was cloned into pBSSK cut with *Eco*RV and *Pst*I resulting in pHYB101. The plasmid was digested with *Hind*III-*Nar*I, treated with Klenow fragment in order to generate blunt ends, and religated, resulting in plasmid pHYB102. The deletion was characterized by nucleotide sequencing. To construct
plasmid pHYB603, the *ApaI-NotI* fragment from pHYB102 was ligated into pBMK1 restricted with *ApaI* and *NotI*.

### 4.1.2.2 Construction of an isogenic *A. pleuropneumoniae* mutant deficient in [NiFe] hydrogenase 2

Plasmid pHYB603 was transformed into *E.coli* β2155 and conjugated into *A. pleuropneumoniae* AP76. Approximately 10 transconjugants were obtained per mating. Kanamycin resistant transconjugants were investigated for plasmid cointegration by PCR analyses using internal primers o34-1f and o34-1r. Colonies containing plasmid cointegrates were used for counterselection in medium containing 10% sucrose. Sucrose resistant colonies were tested for kanamycin resistance on PPLO agar containing kanamycin (25 µg/ml). The sucrose resistant and kanamycin sensitive colonies were further analyzed for presence of the defined deletion.

### 4.1.2.3 Analysis of isogenic [NiFe] hydrogenase 2 deletion mutants

Sucrose resistant and kanamycin sensitive colonies were analyzed to differentiate between wild type and deletion mutants by PCR using primers o34-1f and o34-1r. Ten percent of sucrose resistant colonies showed the expected PCR profile (Fig. 3A). The sequences of the PCR products of *A. pleuropneumoniae* *Appwt* and *App∆hyb* were analyzed and compared. The results showed that the expected deletions had occurred. In addition, the [NiFe] hydrogenase mutant was confirmed by Southern blot analysis. The genomic DNA of *A. pleuropneumoniae* AP76 and *App∆hyb* was digested with *BspDI* and *HindIII* and hybridized using the PCR-product obtained with the primers o34-1f and o34-1r as a probe. A shift of the hybridizing fragment in Southern blot analysis indicated that the [NiFe] hydrogenase gene of *App∆hyb* was deleted as implied by PCR and confirmed nucleotide sequence analysis (Fig. 3B). In order to exclude the rearrangement of chromosomal DNA, pulse-field gel electrophoresis of *ApaI, Ascl* and *NotI* digested DNA was performed and compared to the *A. pleuropneumoniae* wild type (*Appwt*). The restriction fragment pattern of *App∆hyb* was identical to *Appwt* and proved the absence of gross rearrangement of chromosomal DNA (Fig. 3C).
Results

Fig. 2: Construction of plasmid pHYB603
Fig. 2: Construction scheme and physical map of pHYB603. Solid boxed arrows and arrowheads represent the positions and orientations of the important features of the plasmids. The following abbreviations are used: *ColE1 ori*, *ColE1* origin of replication; *mob* RP4, gene encoding mobilizing function originating from plasmid pRP4; *omlA-P*, *omlA* promoter; *sacB*, gene encoding levansucrase conferring sucrose sensitivity; *Km*, kanamycin resistance determinant; *Amp*, ampicillin resistance determinant. The open boxes indicate the restriction sites used in cloning step. Asterisks denote unique restriction enzyme sites in the multiple cloning site of pBMK1.
Fig. 3: Analysis of *A. pleuropneumoniae* (lane 1) and *A. pleuropneumoniae*Δhyb (lane 2). N = negative control for PCR and M size marker. (A) PCR primers with o34-1f and o34-1r (B) Southern blot analysis of *Bsp*DI and *Hind*III digested chromosomal DNA hybridized using the PCR product obtained with primers o34-1f and o34-1r as probe (C) PFGE of *Apal*, *Ascl* and *NotI*-restricted DNA and arrows from PFGE indicate the localization of *hybB* on *A. pleuropneumoniae* genomic map (OSWALD et al. 1999a)
4.1.3 Role of the [NiFe] hydrogenase 2 in an *A. pleuropneumoniae* aerosol infection experiment

*A. pleuropneumoniae Δhyb* (*AppΔhyb*) was used in an aerosol infection model in comparison to the *A. pleuropneumoniae* AP76 wild type strain (*Appwt*). The challenge dose was 1.28 x $10^5$ bacteria for four pigs in the aerosol chamber in the *Appwt* and 1.65 x $10^5$ bacteria in the *AppΔhyb* group. Body temperatures were monitored daily (Fig. 4). Animals challenged with the mutant showed significantly lower increases in body temperatures on days 2 to 6 after infection. One animal from the mutant group died on day 9 post infection due to severe respiratory symptoms. BALF was collected one week before as well as one and three weeks after challenge. The challenge strains were reisolated from BALF on day 7 and on day 21 post infection from several pigs in both challenge groups (Table 5). No consistent difference with respect to the number of *A. pleuropneumoniae* colonies was observed between the groups or between day 7 and day 21.

4.1.3.1 Post mortem examination

The post mortem examination of pigs on day 21 post infection revealed that seven of eight animals challenged with *Appwt* and all animals with *AppΔhyb* had lung lesions. There was no statistically significant difference in lung lesion scores (Fig. 5). The bacteriological examination of tonsils, lung lymph nodes, heart, lung tissue, and pneumatic lesion (if present) revealed that only pigs with lung lesions were culture positive. *A. pleuropneumoniae* was consistently reisolated from surface smears of pneumatic lesions, including sequestered regions showing dense or confluent growth. The mediastinal lymph nodes were culture-positive in one pig in the wild type group and in two pigs in the mutant group. The morphologically intact lung tissue was culture positive in all pigs with lung lesions (Table 5).

4.1.3.2 Humoral immune response

Two different ELISA systems were used to determine the humoral immune response of pigs. One ELISA system was based on detergent extract as solid phase antigen (detergent wash ELISA) and another ELISA was based on recombinant ApxIIA protein (Apx ELISA) as solid phase antigen. On day 7 post infection, an increase in the serum titer in the detergent wash ELISA was seen in the wild type group and mutant groups. A response in the Apx ELISA was
first observed two weeks after infection in both groups. In the AppΔhyb group, a lower immune response than in the Appwt group was seen in the ApxII ELISA at 21 days post infection. However, there was no statistically significant difference between groups in the humoral immune responses determined (Fig. 6).
Fig. 4: Body temperature of pigs over the course of 11 days, day 0 making the day of infection

Asteriks denote statistically significant result (p<0.05).
Fig. 5: Lung lesion score of animals (21 days after infection)

Fig. 6: Humoral immune response of pigs after challenged with parent strain and isogenic mutant strain
<table>
<thead>
<tr>
<th>A. pleuropneumoniae challenge strain</th>
<th>challenge dose (cfu aerosolized)</th>
<th>serological response (Arithmetic mean)</th>
<th>animals with lung lesion</th>
<th>Arithmetic mean of lung lesion score</th>
<th>Number of animals and location of reisolation of A. pleuropneumoniae from BALF at post mortem analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Detergent Wash (Titer)</td>
<td>ApxIIA (Unit)</td>
<td></td>
<td>after challenge</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>day 7</td>
</tr>
<tr>
<td>App wt</td>
<td>$1.28 \times 10^5$</td>
<td>30500</td>
<td>36.2</td>
<td>7/8</td>
<td>7.09</td>
</tr>
<tr>
<td>AppΔhyb</td>
<td>$1.65 \times 10^5$</td>
<td>34742</td>
<td>28.3</td>
<td>7/8</td>
<td>5.47</td>
</tr>
</tbody>
</table>

Table 5: Virulence study of A. pleuropneumoniae Appwt and isogenic mutant (AppΔhyb) in aerosol infection
4.2 The *A. pleuropneumoniae* L-1,2 Propanediol Oxidoreductase gene (*fucO*)

4.2.1 Sequence analysis

A plasmid designated as pRN402 was obtained from screening a genomic library of *A. pleuropneumoniae* by using fragment RN37 obtained by representational difference analysis (RDA) as probe; this RDA fragment was identified in a representational difference analysis using cDNA from *A. pleuropneumoniae* cultures induced by the addition of bronchoalveolar lavage fluid (BALF) from *A. pleuropneumoniae*-infected pigs (BALTES 2002). RDA fragment RN37 is a fragment of 328 bp length and contains a partial ORF that is 68 % identical to L-fucolose 1-phosphate aldolase of *Haemophilus influenzae* (GenBank accession no. P44777) and 56 % identical to L-fucolose 1-phosphate aldolase of *E. coli* (GenBank accession no. P11550). The analysis of plasmid pRN402 revealed that it contains three complete ORFs. The first ORF, 648 bp in length, encodes 215 amino acids of a putative L-fuculose 1-phosphate aldolase with a calculated molecular mass of 23.95 kDa. The predicted amino acid sequence was 72 % identical to L-fucolose 1-phosphate aldolase of *Haemophilus influenzae* and 66 % identical to *E. coli* (CHEN et al. 1989). The second ORF is 82 % identical to L-fucose permease of *Haemophilus influenzae* (GenBank accession no. P44776) and encodes 428 amino acids with a calculated molecular mass of 46.89 kDa. The last ORF encodes a putative L-1,2 propanediol oxidoreductase with 383 amino acids and a calculated molecular mass of 41.21 kDa. The predicted protein has an identity of 67 % to L-1,2 propanediol oxidoreductase of *E. coli* (GenBank accession no. P11549). The nucleotide and amino acid sequences are listed in appendix 8.3.

4.2.2 Construction and characterization of an isogenic *A. pleuropneumoniae* L-1,2 propanediol oxidoreductase mutant

4.2.2.1 Construction of plasmid pFUO604 for the introduction of a L-1,2 propanediol oxidoreductase deletion into *A. pleuropneumoniae* AP76

To construct plasmid pFUO604 (Fig. 7) used for transconjugation, the Scal-HindIII fragment containing the 1152 bp L-1,2 propanediol oxidoreductase gene located on pRN402 was cloned into pBSSK cut with SmaI-HindIII resulting in pFUO103. Plasmid pFUO103 was
Results

partially digested with *XhoI*, treated with Klenow to delete the *XhoI* restriction site in the vector thereby resulting in pFUO104. Plasmid pFUO104 was digested with *PacI*-XhoI, treated with T4 DNA polymerase in order to generate blunt ending, and religated resulting in plasmid pFUO105. The deletion was characterized by nucleotide sequencing. To construct plasmid pFUO604, The *ApaI*-NotI fragment from pFUO104 was ligated into pBMK1 restricted with *ApaI* and *NotI*.

### 4.2.2.2 Construction of an isogenic *A. pleuropneumoniae* mutant deficient in L-1,2 Propanediol Oxidoreductase

Plasmid pFUO604 was transformed into *E.coli* B2155 and conjugated into *A. pleuropneumoniae* AP76. Approximately 5-10 transconjugants were obtained per mating. Kanamycin resistant transconjugants were characterized for plasmid cointegration by PCR using internal primers oRN37-7r and oFUO-2r. Colonies containing plasmid cointegrates were used for counterselection in medium containing 10% sucrose. Sucrose resistant colonies were tested for kanamycin resistance on PPLO agar containing kanamycin (25 µg/ml). The sucrose resistant and kanamycin sensitive colonies were further analyzed for the presence of the defined deletion.

### 4.2.2.3 Analysis of isogenic L-1,2 propanediol oxidoreductase deletion mutants

Sucrose resistant and kanamycin sensitive colonies were analyzed to differentiate between wild type and deletion mutant by PCR using primers oRN37-7r and oFUO-2r. Ten percent of sucrose resistant colonies showed the expected PCR profile (Fig. 8A). The sequence of the PCR products of *A. pleuropneumoniae Appwt* and *AppΔfuo* were analysed and compared. The results showed that the expected deletion had occurred. In order to confirm the oxidoreductase deletion in *AppΔfuo*, the genomic DNA of *A. pleuropneumoniae Appwt* and *AppΔfuo* was digested with *ScaI*-HindIII and *XhoI*-HindIII, blotted and hybridized with the PCR product obtained with primers oRN37-7r and oFUO-2r. The *ScaI* and *HindIII* restriction sites are situated outside of the deletion and, therefore, the respective digests result in a smaller band appearing on the blot (Fig. 8B). The *XhoI* restriction site was used for constructing the deletion. Therefore, a single large band of *XhoI*-HindIII digested *AppΔfuo* DNA appeared on the blot due to the deletion of the *XhoI* restriction site. In addition, PFGE pattern of *ApaI*, *Ascl* and *NotI* of digested DNA were identical in *Appwt* and *AppΔfuo* thereby indicating that no gross chromosomal rearrangement had occurred (Fig. 8C).
Fig. 7: Construction of pFUO604
Fig. 7: Construction scheme and physical map of pFUO604. Solid boxed arrows and arrowheads represent the positions and orientations of the important features of the plasmids. The following abbreviations are used: ColEI ori, ColEI origin of replication; mob RP4, gene encoding mobilizing function originating from plasmid pRP4; omlA-P, omlA promoter; sacB, gene encoding levansucrase conferring sucrose sensitivity; Km, kanamycin resistance determinant; Amp, ampicilin resistance determinant. The open boxes indicate the restriction sites used in cloning step. Asterisks denote unique restriction enzyme sites in the multiple cloning site of pBMK1.
Fig. 8: Analysis of *A. pleuropneumoniae* (lane 1) and *A. pleuropneumoniae*Δ*fuo* (lane 2), N = negative control for PCR and M size marker. (A) PCR primers with oRN37-7r and oFUO-2r (B) Southern blot analysis of Scal/HindIII and XhoI/HindIII digested chromosomal DNA hybridized with probe oRN37-7r/oFUO-2r PCR product (C) PFGE of ApaI, AscI and NotI
4.2.3 Role of L-1,2 propanediol oxidoreductase in an *A. pleuropneumoniae* aerosol infection experiment

Two groups of eight pigs each were infected with *A. pleuropneumoniae* (*Appwt*) strain and *A. pleuropneumoniaeΔfuo* (*AppΔfuo*) strain. Challenge doses for four pigs were $0.33 \times 10^5$ for *Appwt* and $0.69 \times 10^5$ for *AppΔfuo*. Within 1-2 days post infection, most animals developed clinical symptoms typical for an *A. pleuropneumoniae* infection, such as hyperthermia, inappetence and coughing. Body temperatures were monitored daily (Fig. 9). On day 3 post infection, animals infected with *AppΔfuo* were found to have significantly (p<0.05) higher body temperature than animals infected with *Appwt*. One animal from the mutant group died on day 2 post infection due to high fever and severe dyspnoea. Another animal from the mutant group had to be euthanized on day 8 post infection because of severe lameness. At necropsy, the animal showed pleuritis and a small region of adhesion of the lung with the thorax cavity. A second animal from the mutant group was euthanized on day 13 post infection due to severe respiratory symptoms. This animal showed severe pleuritis and sequestered lung lesions at post mortem analysis.

4.2.3.1 Post mortem examination

Upon necropsy 21 days post infection, 4 of the 5 remaining animals challenged with *AppΔfuo* exhibited pleuritis, lung necrosis and sequestration. One animal from the mutant group as well as one animal from the control group was found to have no lung lesions. The difference of lung lesion scores was not statistically significant between both groups (Fig. 10). The challenge strains were consistently reisolated from pneumonic lesions in cultures with surface smears showing dense (+ +) or confluent (+ + +) growth in 5 of 8 pigs infected with *Appwt* and 7 of 8 pigs infected with *AppΔfuo*. From tonsils, reisolation succeeded in 5 animals from *Appwt* group and 3 animals from *AppΔfuo* group. The lung lymph nodes were culture positive in 4 animals from *Appwt* group and 6 animals from *AppΔfuo* group (Table 6). The raw data for this challenge experiment can be obtained from table 8 in appendix.
4.2.3.2 Systemic immune response

Systemic immune response was determined with two ELISA systems, using recombinant ApxIIA protein or detergent extract of *A. pleruopneumoniae*-cultures grown under iron restricted condition as solid-phase antigen. All animals challenged with *App*wt or *AppΔfuo* showed a humoral immune response in both systems at 21 days post infection and no statistically significant difference was observed between the groups (Fig. 11).
Fig. 9: Body temperature of pigs over the course of 14 days, day 0 making the day of infection.
Asterisks denote statistically significant result (p<0.05).
Results

Fig. 10: Lung lesion score of animals (21 days after infection)

Fig. 11: Humoral immune response of pigs after challenged with parent strain (Appwt) and isogenic mutant strain (AppΔfuo)
Table 6: Virulence study of *A. pleuropneumoniae* Appwt and isogenic mutant (*AppΔfuo*) in aerosol infection

<table>
<thead>
<tr>
<th>A. pleuropneumoniae challenge strain</th>
<th>challenge dose (cfu aerosolized)</th>
<th>serological response (Arithmetic mean)</th>
<th>animals with lung lesion</th>
<th>Arithmetic mean of lung lesion score</th>
<th>Number of animals and location of reisolation of <em>A. pleuropneumoniae</em> at post mortem analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Detergent Wash (Titer)</td>
<td>ApxIIA (Unit)</td>
<td></td>
<td>tonsil  lymph node  heart  lung</td>
</tr>
<tr>
<td>Appwt</td>
<td>0.33 x 10⁵</td>
<td>7085</td>
<td>18.86</td>
<td>7/8</td>
<td>7.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5       4       4       5</td>
</tr>
<tr>
<td>AppΔfuo</td>
<td>0.69 x 10⁵</td>
<td>16960</td>
<td>12.6</td>
<td>7/8</td>
<td>6.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3       6       5       7</td>
</tr>
</tbody>
</table>
5 Discussion

A main concern in the control of *A. pleuropneumoniae* infection is the occurrence of carrier animals even after vaccination (FENWICK and HENRY 1994; HAESEBROUCK et al. 1997). Since natural infection with *A. pleuropneumoniae* can prevent colonization upon reinfection and has been shown to provide cross-protection (NIELSEN 1984), an attenuated live vaccine that allows discrimination of immunized and infected animals would be desirable. Attenuation can be achieved by deletion of a gene that plays a role in virulence. As it has been shown that certain genes are expressed by *A. pleuropneumoniae* only in vivo (HENNIG et al. 1999), a subtracted *A. pleuropneumoniae* cDNA library containing genes expressed upon the addition of BALF to the culture medium was constructed (BALTES 2002). Many genes were identified in this experiment, among them, the catalytic subunit of dimethylsulfoxide reductase (*dmsA*) (BALTES 2002) and a novel ferric compound receptor (*fhuA*) (BALTES et al. 2003). Isogenic mutants were constructed to investigate the possible role of these genes in virulence in an aerosol infection model. In a challenge experiment both strains were shown to be attenuated in acute disease.

Parts of the [NiFe] hydrogenase 2 subunit gene (*hybB*) and L-fuculose 1-phosphate aldolase gene (*fucA*) were identified from the same differential screening experiment. For further analyses, two large fragments containing the entire genes were obtained from a genomic library of *A. pleuropneumoniae* by using the cDNA fragments as probes. One fragment designated as RN400 revealed a complete ORF encoding for a putative *hybB* gene encoding a [NiFe] hydrogenase 2 probable cytochrome b subunit in DNA sequence analysis. The putative *hybA*, *hybC*, *hybD*, *hybE*, *hybF*, and *hybG* ORFs were identified in the vicinity of the *hybB* ORF in a BLAST search on the incomplete genomic sequence of *A. pleuropneumoniae* serotype 7. These genes are arranged in one operon in the order *hybABCDEFG*, with the *hybF* ORF being arranged in the opposite direction to the others. In *E. coli*, the [NiFe] hydrogenase 2 gene is encoded by an operon *hybOABCDEFG* (MENON et al. 1994) and the HybB protein of *E. coli* has sequence similarity to b-type cytochrome (MENON et al. 1994; SARGENT et al. 1998).
By nucleotide sequence analysis another fragment, designated as RN402, was found to contain three complete ORFs. These ORFs encode a putative L-fuculose 1-phosphate aldolase (FucA), a L-fucose permease (FucP) and a L-1,2 propanediol oxidoreductase (FucO), respectively. The organization of the fucose regulon homologue in *A. pleuropneumoniae* differs from that of the *E. coli* fucose regulon (CHEN et al. 1989); thus the fucose regulon of *E. coli* contains at least two operons, namely, the *fucAO* and *fucPIK* operons. In *A. pleuropneumoniae*, a fucose isomerase (FucI) and a fucose kinase (FucK) were not identified in this study.

Both operons identified have been shown to be expressed primarily under anaerobic conditions in *E. coli* (COCKS et al. 1974; BALLANTINE and BOXER 1985; MENON et al. 1994; RICHARD et al. 1999). Previously, BALTES (2002) had identified the *A. pleuropneumoniae* DMSO reductase subunit DmsA, which also is expressed primarily under anaerobic conditions, and an isogenic *A. pleuropneumoniae dmsA* mutant was found to be attenuated in an aerosol infection experiment. Based on these findings, it was hypothesized that an impairment of [NiFe] hydrogenase 2 or L-1,2 propanediol oxidoreductase function might reduce persistence of *A. pleuropneumoniae* under anaerobic conditions as they occur in necrotic tissues and, thereby, decrease the virulence. Although neither enzyme has been associated with virulence to date, an impairment of anaerobic metabolism as a strategy to reduce virulence has been successfully employed in *Mycobacterium (M.) bovis* BCG. This organism, as an obligate aerobe, survives inside granulomatous lung lesions, and an *M. bovis* BCG mutant lacking anaerobic nitrate reductase was attenuated in immunodeficient mice (WEBER et al. 2000; FRITZ et al. 2002).

Based on the hypothesis that an impairment of the anaerobic metabolism might reduce the persistence of bacteria within the host, [NiFe] hydrogenase 2 and L-1,2 propanediol oxidoreductase deficient *A. pleuropneumoniae* mutants were constructed via homologous recombination, using a suicide vector (pBMK1) and a selection/counterselection procedure with kanamycin and sucrose, respectively (OSWALD et al. 1999b), resulting in two isogenic deletion mutants *A. pleuropneumoniae Δhyb* and *A. pleuropneumoniae Δfuo*; their virulence were examined in an aerosol infection model (JACOBSEN et al. 1996; BALTES et al. 2001).
For the [NiFe] hydrogenase 2, it was found that reisolation rates, lung lesion scores and antibody titers were not significantly different from the wild type, whereas the body temperatures of the animals infected with the deletion mutant \textit{A. pleuropneumoniae App\textsuperscript{∆hyb}} were significantly lower on days 3, 4, 5 and 6 post infection; also, these animals had a reduced immune response on days 14 and 21 post infection. This milder course of disease combined with the decreased humoral immune response may be due to a decrease in \textit{in vivo} growth of \textit{A. pleuropneumoniae} caused by the absence of hydrogenase activity in the early stage of infection. However, contrary to the initial hypothesis, long-term survival of the pathogen in the host does not appear to be affected by the deletion. In \textit{E. coli}, [NiFe] hydrogenase 2 is involved in the anaerobic hydrogen metabolism and in the reduction of fumarate to succinate by contributing electrons from hydrogen oxidation to the fumarate reductase (SAWERS and BOXER 1985; MENON et al. 1990), the enzyme has not been associated with virulence. The finding that only a slight attenuation of \textit{A. pleuropneumoniae ∆hyb} was observed in the aerosol infection experiment implies that a residual HybB function might have been present during the course of the infection. Such a residual function might have occurred because of the in-frame deletion in the hybB gene constructed is only 169 bps in length, whereas the entire hybB gene is 1182 bps in total length. Further, the H-X-X-H motif essential for function, which is conserved in cytochrome b type proteins (MENON et al. 1990, 1994), was not deleted in \textit{A. pleuropneumoniae ∆hyb}. Alternatively, other hydrogenases could have compensated HybB function; thus, \textit{E. coli} possesses three different hydrogenase enzymes, and all three are expressed under anaerobic condition (MENON et al. 1990, 1994; BÖHM et al. 1990).

Interestingly, the consensus sequence for the twin arginine translocation (TAT) pathway was identified in the signal peptide sequence of hybA. Remarkably, in the same differential screening experiment that led to the identification of the hyb operon in \textit{A. pleuropneumoniae}, the gene encoding another TAT-dependent protein, the DMSO reductase subunit DmsA, was identified, and its deletion was found to cause an attenuation of \textit{A. pleuropneumoniae} (BALTES 2002). These results implied that TAT-dependent proteins might play a role in virulence of \textit{A. pleuropneumoniae} as it has recently been shown for \textit{Pseudomonas aeruginosa}. In this organism, TAT-dependent phospholipases as well as iron siderophore biosynthesis and uptake proteins have been associated with virulence (OCHSNER et al.}
Thus, it was demonstrated that a deletion of the \textit{tatC} gene, a component of the TAT machinery, effectively attenuated \textit{Pseudomonas aeruginosa} in a rat lung model.

The role of the second gene identified, encoding the L-1,2 propanediol oxidoreductase, was also investigated in the aerosol infection model using an isogenic deletion mutant \textit{A. pleuropneumoniae}\textsubscript{\textit{\textDelta fuo}}. Surprisingly, the isogenic mutant strain \textit{App}\textsubscript{\textDelta fuo} remained fully virulent in the challenge experiment. This indicated that the L-1,2 propanediol oxidoreductase is not required for virulence in \textit{A. pleuropneumoniae}. These findings also showed that the anaerobic utilization of L-fucose is not important for survival and growth of \textit{A. pleuropneumoniae} in the necrotic lung tissues thereby implying that \textit{A. pleuropneumoniae} uses other anaerobic pathways to survive within the host.

On the other hand, von BISMARCK et al. (2001) reported that fucose could be used to eliminate \textit{Pseudomonas aeruginosa} from a patient suffering from airway infection by the competitive inhibition of its specific lectin receptor (PA-IIL). This fucose binding lectin (PA-IIL) is produced by \textit{Pseudomonas aeruginosa} and used for binding to host tissue (GILBOA-GARBER 1982). Further, JEFCOAT et al. (2001) demonstrated that L-fucose levels were increased in the mucins of horses suffering from chronic airway infection. However, the reisolation rates of \textit{A. pleuropneumoniae} AP76 wild type were not different from those of \textit{A. pleuropneumoniae}\textsubscript{\textit{\textDelta fuo}}. This finding implies that either fucose is not involved in the binding \textit{A. pleuropneumoniae} to the host or that the L-1,2 propanediol oxidoreductase cannot interfere with fucose binding.

In summary, an isogenic [NiFe] hydrogenase 2 mutant and a L-1,2 propanediol oxidoreductase mutant without antibiotic marker were constructed by using a 'single step transconjugation system'. Subsequently, their possible role in virulence was examined in an aerosol infection experiment. The L-1,2 propanediol oxidoreductase was found to not be required for virulence whereas the disease caused by an \textit{A. pleuropneumoniae}\textsubscript{\textit{\textDelta hybB}} mutant was less severe than that induced by the parent strain, even though long-term survival of the pathogen did not appear to be affected. Therefore, the \textit{hybB} deletion may be useful for the construction of a live vaccine strain carrying multiple deletions.
6 Summary/Zusammenfassung

Construction and characterization of genetically defined metabolic mutants of *Actinobacillus pleuropneumoniae*

(Kyaw Sunn)

*Actinobacillus pleuropneumoniae* is a major threat to the swine industry due to the pathogen's ability to persist in host tissues even after vaccination with widely used bacterins, and due to the lack of cross-serotype protection. Since natural infection can provide cross-serotype protection, an attenuated live marker vaccine strain may be a useful tool.

Parts of the genes encoding a putative [NiFe] hydrogenase 2 subunit (*hybB*) and the L-fuculose 1-phosphate aldolase (*fucA*) were identified from a subtracted *A. pleuropneumoniae* AP76 cDNA library obtained after induction of *A. pleuropneumoniae* cultures with BALF from animals infected with *A. pleuropneumoniae*. For further analyses, large fragments containing the entire genes were isolated from a genomic library by hybridizing with the cDNA fragments as probes.

Nucleotide sequencing analyses of one fragment, designated as RN400, revealed a complete ORF encoding the entire [NiFe] hydrogenase 2 (*hybB*). In a BLAST search on the incomplete genomic sequence of *A. pleuropneumoniae* serotype 7 with the *A. pleuropneumoniae* AP76 *hybB* ORF, a putative *hybABCDEFG* operon was identified; the putative *A. pleuropneumoniae* HybA protein contains a recognition sequence for the twin arginine translocation (TAT) pathway. The second fragment, designated as RN402, contained three complete ORFs as assessed by nucleotide sequence analysis. These ORFs encode a putative L-fuculose 1-phosphate aldolase (*fucA*), an L-fucose permease (*fucP*), and an L-1,2 propanediol oxidoreductase (*fucO*) respectively.

Based on hypothesis that adaptation of *A. pleuropneumoniae* to anaerobic conditions, as they occur in necrotic lung lesion, may play role in *A. pleuropneumoniae* virulence and persistence in the host, two isogenic mutants with a deletion in the *hybB* and the *fucO* gene, respectively, were constructed and examined in an aerosol infection model. The *A. pleuropneumoniae* mutant with a deletion in the *fucO* gene remained fully virulent in the challenge experiment,
implying that the L-1,2 propanediol oxidoreductase is not required for virulence in \textit{A. pleuropneumoniae}. The deletion mutant \textit{A. pleuropneumoniae} $\Delta$hyb was shown to be slightly attenuated in acute disease. Therefore, the hybB deletion may be useful for the construction of a live vaccine strain carrying multiple deletions.
Erstellung und Charakterisierung genetisch definierter isogener Stoffwechselmutanten von *Actinobacillus pleuropneumoniae* (Sunn, Kyaw)


Basierend auf der Hypothese, dass eine Beeinträchtigung des anaeroben Stoffwechsels die Vermehrung von *A. pleuropneumoniae* im Tier verlangsamen und möglicherweise die Persistenzfähigkeit reduzieren könnte, wurden zwei isogene Mutanten mit einer Deletion im *hybB* und *fucO* Gen konstruiert und in einem Aerosolinfektionsmodell überprüft. *A. pleuropneumoniae* mit einer Deletion im *fucO* Gen war im Belastungversuch nicht attenuiert. Die Deletionsmutante *A. pleuropneumoniaeΔhyb* zeigte sich leicht attenuiert; insbesondere war die Erhöhung der Körpertemperatur bei den infizierten Tieren signifikant geringer als bei den Kontrolltieren. Somit könnte eine *hybB*-Deletion in Kombination mit anderen bereits
bekannten Deletionen in virulenzassozierten Genen bei der Konstruktion eines Markervakzinestamms von Nutzen sein.
7 References

Pseudomonas aeruginosa II lectin stops human ciliary beating: therapeutic implication of fucose.

ALTSCHUL, S. F., W. GISH, W. MILLER, E. W. MYERS, and D. J. LIPMAN (1990):
Basic local alignment search tool.

Isolation and molecular characterization of spontaneously occurring cytolysin-negative mutants of Actinobacillus pleuropneumoniae serotype 7.
Infect. Immun. 59, 4110-4116.

Nickel containing isoenzymes from anaerobically grown Escherichia coli K-12.

Isolation and characterization of a soluble active fragment of hydrogenase isoenzyme 2 from the membranes of anaerobically grown Escherichia coli.
J. Biochem. 156, 277-284.

Actinobacillus pleuropneumoniae iron transport and urease activity: effects on bacterial virulence and host immune response.
Infect. Immun. 69, 472-478.

The role of iron in Actinobacillus pleuropneumoniae infection: Identification and in vivo characterization of virulence-associated genes.
Tierärztliche Hochschule Hannover, PhD-Thesis
*Actinobacillus pleuropneumoniae* serotype 7 siderophore receptor FhuA is not required for virulence.  

Isolation of a mutation resulting in constitutive synthesis of L-fucose catabolic enzymes.  

Role of lipopolysaccharides adherence of *Actinobacillus pleuropneumoniae* to porcine tracheal rings.  
Infect. Immune. 58, 3523-3530.

Lipopolysaccharides of *Actinobacillus pleuropneumoniae* bind pig hemoglobin.  

BERKS, B. C. (1996):  
A common export pathway for proteins binding complex redox cofactors?  

BERKS, B. C., F. SARGENT, and T. PALMER (2000):  
The Tat protein export pathway.  

Pleuropneumonia in swine associated with a urease-negative variant of Actinobacillus pleuropneumoniae serotype 1.  

BIRNBOIM, H. C. and J. DOLY (1979):  
A rapid alkaline lysis extraction for screening recombinant plasmid DNA.  
Successful treatment of *Pseudomonas aeruginosa* respiratory tract infection with a sugar solution-- a case report on a lectin based therapeutic principle.

BÖHM, R., M. SAUTER, and A. BÖCK (1990):
Nucleotide sequencing and expression of an operon in *Escherichia coli* coding for formate hydrogenase components.

Protective local and systemic antibody responses of swine exposed to an aerosol of *Actinobacillus pleuropneumoniae* serotype 1.
Infect. Immun. **60**, 479-484.

BOSSE, J. T., and J. I. MACINNES (2000):
Urease activity may contribute to the ability of *Actinobacillus pleuropneumoniae* to establish infection.

BYRD, W., and S. KADIS (1989):
Structures and sugar compositions of lipolysaccharides isolated from seven *Actinobacillus pleuropneumoniae* serotypes.

BYRD, W., B. G. HARMON, and S. KADIS (1992):
Protective efficacy of conjugate vaccines against experimental challenge with porcine *Actinobacillus pleuropneumoniae*.

Molecular analysis of the *Actinobacillus pleuropneumoniae* RTX-Toxin-III gene cluster.
The organization of the fuc regulon specifying L-fucose dissimilation in Escherichia coli as
determined by gene cloning.
J. Bacteriol. 210, 331-337.

Constitutive activation of the fucAO operon and silencing the divergently transcribed fucPIK
operon by IS5 element in Escherichia coli mutants selected for growth on L-1,2-propanediol.

CHIERS, K., O. I. VAN, P. DE LAENDER, R. DUCATELLE, S. CAREL, and F.
HAESEBROUCK (1998):
Effects of endobronchial challenge with Actinobacillus pleuropneumoniae serotype 9 of pigs
vaccinated with inactivated vaccines containing the Apx toxins.

Evolution of L-1,2-propanediol catabolism in Escherichia coli recruitment of enzymes for L-
fucose and L-lactate metabolism.

COLLINS, C. M., and S. E. D'ORAZIO. (1993):
Bacterial ureases: structure, regulation of expression and role in pathogenesis.
Mol. Microbiol. 9, 907-913.

CRUJISEN, T., L. A. VAN LEENGOED, M. HAM-HOFFIES, and J. H. VERHEIJDEN
(1995):
Convalescent pigs are protected completely against infection with a homologous
Actinobacillus pleuropneumoniae strain but incompletely against a heterologous-serotype
strain.
Infect. Immun. 63, 2341-2343.

DABAN, M., A. MEDRANO, and E. QUEROL (1996):
Cloning, sequencing and expression of the transferrin-binding protein 1 gene from
Actinobacillus pleuropneumoniae.
DEHIO, C., and M. MEYER (1997):
Maintenance of broad-host-range incompatibility group P and group Q plasmids and transposition of Tn5 in *Bartonella henselae* following conjugal plasmid transfer from *Escherichia coli*.

DEVENISH, J., S. ROSENDAL, and J. T. BOSSE (1990):
Humoral antibody response and protective immunity in swine following immunization with the 104-kilodalton hemolysin of *Actinobacillus pleuropneumoniae*.

DIDIER, P. J., L. PERINO, and J. URBANCE (2002):
Porcine *Haemophilus pleuropneumonia*: microbiologic and pathologic findings.

Prokaryotic utilization of the twin-arginine translocation pathway: a genomic survey.

Influence of *Actinobacillus pleuropneumoniae* serotype 2 and its cytolsins on porcine neutrophilchemiluminescence.

DOM, P., F. HAESEBROUCK, and P. DE BAETSELIER (1992):
Stimulation and suppression of the oxygenation activity of porcine pulmonary alveolar macrophage by *Actinobacillus pleuropneumoniae* and its metabolites.

DRAPAL, N., and A. BÖCK (1998):
Interaction of the hydrogenase accessory protein HypC with HypE, the large subunit of *Escherichia coli* hydrogenase 3 during enzyme maturation.
DROSS, F., V. GEISLER, R. LENGER, F. THEIS, T. KRAFFT, F. FAHRENHOLZ, E.
The quinone-reactive [NiFe] hydrogenase of Wolinella succinogenes.

FENWICK, B., and S. HENRY (1994):
Porcine pleuropneumonia.

FREY, J. and J. NICOLET (1988):
Purification and partial characterization of a hemolysin produced by Actinobacillus
pleuropneumoniae type strain 4074.
FEMS Microbiol. lett. 55, 41-46.

FREY, J. and J. NICOLET (1990):
Hemolysin patterns of Actinobacillus pleuropneumoniae.

FREY, J., J. T. BOSSE, Y. F. CHANG, J. M. CULLEN, B. FENWICK, G.-F. GERLACH, D.
GYGI, F. HAESEBROUCK, T. J. INZANA, and R. JANSEN. (1993):
Actinobacillus pleuropneumoniae RTX-toxins: uniform designation of haemolysins,
cytolysins, pleurotoxin and their genes.
J. Gen. Microbiol. 139, 1723-1728.

Virulence in Actinobacillus pleuropneumoniae and the RTX toxins.

FREY, J., M. BECK, and J. NICOLET (1996):
Typizierung der Apx-toxin-gene von Actinobacillus pleuropneumoniae mittels PCR.

Crystal structure of the hydrogenase maturating endopeptidase HybD from Escherichia coli
FRITZ, C., S. MAASS, A. KREFT, and F. C. BANGE (2002):
Dependence of *Mycobacterium bovis* BCG on anaerobic nitrate reductase for persistence is tissue specific.
Infect. Immun. 70, 286-291.

Biochemical evidence for a conserved interaction between bacterial transferrin binding protein A and transferrin binding protein B.
Microb. Pathog. 24, 75-87.

A genetically-defined riboflavin auxotroph of *Actinobacillus pleuropneumoniae* as a live attenuated vaccine.
Vaccine. 18, 2867-2877.

Antibody- and cell-mediated immune responses of *Actinobacillus pleuropneumoniae*-infected and bacterin-vaccinated pigs.

Specificity of the fucose-binding lectin of *Pseudomonas aeruginosa*.

A 24-kDa cloned zinc metalloprotease from *Actinobacillus pleuropneumoniae* is common to all serotypes and cleaves gelactin *in vitro*.

Cloning and expression of a transferrin-binding protein from *Actinobacillus pleuropneumoniae*.
Infect. Immun. 60, 892-898.
GHALAMBOR, M. A. and E. C. HEATH (1962):  
The enzymatic cleavage of L-fuculose 1-phosphate.  
J. Biol. Chem. 237, 2427-2433.

Pseudomonas aeruginosa lectins.  


A novel strategy for protective Actinobacillus pleuropneumoniae subunit vaccines: detergent extraction of cultures induced by iron restriction.  
Vaccine. 19, 966-975.

Identification and characterization of a porcine-specific transferrin receptor in Actinobacillus pleuropneumoniae.  

GONZALEZ, G. C., R. H. YU, P. R. ROSTECK JR., and A. B. SCHRYVERS (1995):  
Sequence, genetic analysis, and expression of Actinobacillus pleuropneumoniae transferrin receptor genes.  
Microbiology. 141, 2405-2416.

GREEN, M. and S. S. COHEN (1956):  
The enzymatic conversion of L-fucose to L-fuculose.  
J. Biol. Chem. 219; 557-568.

Functional analysis of the Ca$^{2+}$-regulated hemolysin I operon of Actinobacillus pleuropneumoniae serotype 1.  
Infect. Immun. 60, 3059-3064.
Disruption of the fucose pathway as a consequence of genetic adaptation to propanediol as a carbon source in *Escherichia coli*.
J. Bacteriol. 126, 1166-1172.

*Actinobacillus pleuropneumoniae* infections in pigs: the role of virulence factors in pathogenesis and protection.
Vet. Microbiol. 58, 239-249.

HANNAN, P. C., B. S. BHOQAL, and J. P. FISH (1982):
Tylosin tartrate and tiamutilin effects on experimental piglet pneumonia induced with pneumonic pig lung homogenate containing mycoplasmas, bacteria and viruses.

HEANT, E. C., and M. A. GHALAMBOR (1962):
The purification and properties of L-fuculose kinase.
J. Biol. Chemistry. 237, 2423-2426.

Serotype identification of *Actinobacillus pleuropneumoniae* by arbitrarily primed polymerase chain reaction.

Downregulation of a protective *Actinobacillus pleuropneumoniae* antigen during the course of infection.
Microb Pathogenesis. 26, 53-63.

Intramuscular immunization with genetically inactivated (ghosts) *Actinobacillus pleuropneumoniae* serotype 9 protects pigs against homologous aerosol challenge and prevents carrier state.
Vaccine. 18, 2945-2955.
Cystic fibrosis epithelial cells have a receptor for pathogenic bacteria on their apical surface.

Virulence properties and protective efficacy of the capsular polymer of *Haemophilus (Actinobacilus) pleuropneumoniae* serotype 5.

INZANA, T. J. (1991):
Virulence properties of *Actinobacillus pleuropneumoniae*.
Microb. Pathog. 11, 305-316.

JACOBSEN, J., J. P. NIELSEN, and R. NIELSEN (1996):
Comparison of virulence of different *Actinobacillus pleuropneumoniae* serotypes and biotypes using an aerosol infection model.

Bacterial ghosts as vaccine candidates for veterinary applications.
J. Control Release. 85, 17-25.

Structural analysis of the *Actinobacillus pleuropneumoniae*-RTX-toxin I (ApxI) operon.
Infect. Immun. 61, 3688-3695.

Genetic map of the *Actinobacillus pleuropneumoniae* RTX-toxin (Apx) operons:
characterization of the ApxIII operons.

Persistent mucin glycoprotein alterations in equine recurrent airway obstruction.
Antigenic differences within Actinobacillus pleuropneumoniae serotype 1.

Identification of hemolytic and cytotoxic proteins of Actinobacillus pleuropneumoniae by use
of monoclonal antibodies.
Infect. Immun. 59, 3079-3085.

Production of Apx toxins by field strains of Actinobacillus pleuropneumoniae and
Actinobacillus suis.

KAMP, E. M., N. STOCKHOFE-ZURWIEDEN, L. A. VAN LEENGOED, and M. A.
SMITS (1997):
Endobronchial inoculation with Apx toxins of Actinobacillus pleuropneumoniae leads to
pleuropneumonia in pigs.
Infect Immun. 65, 4350-4354.

KROLL, J. S., P. R. LANGFORD, E. WILKS, and A. D. KEIL (1995):
Bacterial [Cu,Zn]-superoxide dismutase: phylogenetically distinct from the eukaryotic
enzyme, and not so rare after all.
Microbiology. 141, 2271-2279.

KUME, K., T. NAKAI, and A. SAWATA (1984):
Isolation of Haemophilus pleuropneumoniae from the nasal cavities of healthy pigs.

Identification of hemolysin from actinobacillus pleuropneumoniae and characterization of ist
channel properties in planar phospholipid bilayers.
J. Biol. Chem. 264, 13559-13564.
LANGFORD, P. R., B. M. LOYDTS, and J. S. KROLL (1996):
Cloning and molecular characterization of Cu,Zn superoxide dismutase from *Actinobacillus pleuropneumoniae*.
Infect. Immun. 64, 5035-5041.

A novel enzyme-linked immunosorbent assay using the recombinant *Actinobacillus pleuropneumoniae* ApxII antigen for diagnosis of pleuropneumonia in pig herds.
Clin Diagn Lab Immunol. 6, 630-632.

Sequential study of lesion development in experimental *Haemophilus pleuropneumoniae*.
Res. Vet. Sci. 42, 204-212.


*Neisseria meningitidis* expressing transferrin binding proteins of *Actinobacillus pleuropneumoniae* can utilize porcine transferrin for growth.

Prevention and control of *Actinobacillus (Haemophilus) pleuropneumoniae* infection in swine: A review.

MATTHEW, P. R. and I. H. PATTINSON (1961):
The identification of a *Haemophilus*-like organism associated with pneumonia and pleurisy in the pig.
J. Comp. Pathol. 71, 44-52.

Cloning and sequencing of a putative *Escherichia coli* [NiFe] hydrogenase-1 operon containing six operon reading frames.

Mutational analysis and characterization of the *Escherichia coli hya* operon, which encodes [NiFe] hydrogenase 1.
J. Bacteriol. 173, 4851-4861.

Cloning, sequencing, and mutational analysis of the *hyb* operon encoding *Escherichia coli* hydrogenase 2.
J. Bacteriol. 176(14), 4416-4423.

Identification and serotyping of *Haemophilus pleuropneumoniae* by coagulation test.

MITTAL, K. R (1990):
Cross-reaction between *Actinobacillus pleuropneumoniae* strains serotype 1 and 9.

TonB-dependent iron acquisition: mechanisms of siderophore-mediated active transport.
MOLLER, K., L. V. ANDERSEN, G. CHRISTENSEN, and M. KILIAN (1993):
Optimization of the detection of NAD dependent *Pasteruriellaceae* from the respiratory tract of slaughterhouse pigs.

A targeted mutagenesis system for *Actinobacillus pleuropneumoniae*.
Gene. 165, 61-66.

Secreted proteases from *Actinobacillus pleuropneumoniae* serotype 1 degrade porcine gelatin, hemoglobin and immunoglobulin A.

NICOLET, J. (1988):
Taxonomy and serological identification of *Actinobacillus pleuropneumoniae*.

NICOLET, J. (1992):

NIELSEN, R. (1979):
*Haemophilus parahaemohyliticus* serotypes serological response.

*Haemophilus pleuropneumoniae* serotypes cross-protection experiments.

NIELSEN, R. (1985):
*Haemophilus pleuropneumoniae (Actinobacillus pleuropneumoniae).* Serotypes 8, 3 and 6. Serological response and cross immunity in pigs.


Structural characteristics of the antigenic capsular polysaccharides and lipopolysaccharides involved in the serological classification of *Actinobacillus pleuropneumoniae* strains.
Serodiag. Immun. in Infect. Dis. 4, 299-308.

Transfer of *Haemophilus pleuropneumoniae* and Pasteurella Haemolytica-like organism causing porcine necrotic pleuropneumonia to the genus *Actinobacillus* (*Actinobacillus pleuropneumoniae* comb. nov.) On the basis of phenotypic and deoxyribonucleic acid relatedness.


Molecular investigation of the role of ApxI and ApxII in the virulence of *Actinobacillus pleuropneumoniae* serotype 5.
Microb. Pathog. 18, 197-209.

Isolation and identification of a putative transferrin receptors from *Actinobacillus pleuropneumoniae* biotype I.

Transcriptional regulation in response to oxygen and nitrate of the operons encoding the [NiFe] hydrogenases 1 and 2 of *Escherichia coli*.
Microbiology. 145, 2903-3012.

Isolation and characterization of mini-Tn10 lipopolysaccharide mutants of *Actinobacillus pleuropneumoniae* serotype 1.
ROBINSON, C., and A. BOLHUIS (2001):
Protein targeting by the twin-arginine translocation pathway.

ROSSI-CAMPOS, A., C. ANDERSON, G.-F. GERLACH, S. KLASHINSKY, A. A.
POTTER, and P. J. WILLSON (1992):
Immunization of pigs against *Actinobacillus pleuropneumoniae* with two recombinant protein preparations.
Vaccine. 10, 512-518.

Characterization of a protease from *Escherichia coli* involved in hydrogenase maturation.

Experimental reproduction of acute lesions of procine pleuropneumonia with a hemolysin-deficient mutant of *Actinobacillus pleuropneumoniae*.

*Actinobacillus* species and their role in animal disease.
Vet. J. 159, 18-36.

SAMBROOK, J. E., F. FRITSCH and T. MANIATIS (1989):

Reassignment of the gene encoding the *Escherichia coli* hydrogenase 2 small subunit--identification of a soluble precursor of the small subunit in a *hypB* mutant.
Eur J Biochem. 255, 746-54.

Differential expression of hydrogenase isoenzymes in *Escherichia coli* K-12: evidence for a third isoenzyme.
SCHALLER, A., R. KUHN, P. KUHNERT, J. NICOLET, T. J. ANDERSON, J. I.
MACINNES, R. P. SEGERS, and J. FREY (1999):
Characterization of apxIVA, a new RTX determinant of Actinobacillus pleuropneumoniae.
Microbiology. 145, 2105-2116.

Haemophilus pleuropneumoniae infection in swine: a review.

Endothelial cytotoxicity of Actinobacillus pleuropneumoniae.

SHEA, R. J., and M. H. MULKS (2002):
ohr, Encoding an organic hydroperoxide reductase, is an in vivo-induced gene in
Actinobacillus pleuropneumoniae.
Infect. Immun. 70, 794-802.

SHEEHAN, B. J., P. R. LANGFORD, A. N. RYCROFT,, and J. S. KROLL (2000):
[Cu,Zn]-Superoxide Dismutase Mutants of the Swine Pathogen Actinobacillus
pleuropneumoniae Are Unattenuated in Infections of the Natural Host.
Infect. Immun. 68, 4778-4781.

SHOPE, R. R. (1964):
Porcine contagious pleuropneumonia I. Experimental transmission, etiology and pathology J.
Exp. Med. 119, 357-358.

Ferrous-activated nicotanimide adenine dinucleotide-linked dehydrogenase from a mutant of
Escherichia coli capable growth on 1,2 propanediol.
J. Bacteriol. 98, 87-95.

Pathogenicity of RTX toxin mutants of Actinobacillus pleuropneumoniae: results in vivo
studies.
Proc. International Pig Vet. Society Congress, Boloogna, Italy, 189
Bacterial ghosts: non-living candidate vaccines.

TASCON, R. I., J. A. VAZQUEZ-BOLAND, C. B. GUTIERREZ-MARTIN, I.
The RTX haemolysins ApxI and ApxII are major virulence factors of the swine pathogen
Actinobacillus pleuropneumoniae: evidence from mutational analysis.

Actinobacillus pleuropneumoniae iron transport: a set of exbBD genes is transcriptionally
linked to the thpB gene and required for utilization of transferrin-bound iron.

Construction of an Actinobacillus pleuropneumoniae serotype 2 prototype live negative-
marker vaccine.
Infect. Immun. 70, 7120-7125.

Airborne transmission of Actinobacillus pleuropneumoniae and porcine reproductive and
respiratory syndrome virus in nursery pigs.

Role of Haemophilus pleuropneumoniae lipopolysaccharide endotoxin in the pathogenesis of
porcine Haemophilus pleuropneumoniae.

VOLBEDA, A., M. H. CHARON, C. PIRAS, E. C. HATCHIKIAN, M. FREY, and J. C.
FONTECILLA-CAMPS (1995):
Crystal structures of the nickel-iron hydrogenase from Desulfovibrio gigas.
Nature. 373, 580-587.
Involvement of the twin-arginine translocation system in protein secretion via the type II pathway.  
EMBO Journal, 20, 6735-6741.

Resistance of Actinobacillus pleuropneumoniae to bactericidal antibody and complement is mediated by capsular polysaccharide and blocking antibody specific for lipopolysaccharide.  
J. Immunol. 153, 2110-2121

Cloning and mutagenesis of a serotype-specific DNA region involved in encapsulation and virulence of Actinobacillus pleuropneumoniae serotype 5a: concomitant expression of serotype 5a and 1 capsular polysaccharides in recombinant A. pleuropneumoniae serotype 1  
Infect. Immun. 66, 3326-3336.

Anaerobic nitrate reductase (narGHJI) activity of Mycobacterium bovis BCG in vitro and its contribution to virulence in immunodeficient mice.  

A novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins.  
Cell. 93, 93-101.

Characterization of a large transferrin-binding protein from Actinobacillus pleuropneumoniae serotype 7.  
Microbial hydrogenases. primary structure, classification, signatures and phylogeny.
FEMS Microbiol Rev. 10, 243-269.

Membrane targeting and translocation of bacterial hydrogenases.
Appendix

8 Appendix

8.1 Chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier and Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar</td>
<td>OXOID, Wesel</td>
</tr>
<tr>
<td>Agarose</td>
<td>APPLIGENE, Illkirch, France</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>ROTH, Karlsruhe</td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td>ROTH, Karlsruhe</td>
</tr>
<tr>
<td>Ammonium acetate</td>
<td>SIGMA, Deisenhofen</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>SIGMA, Deisenhofen</td>
</tr>
<tr>
<td>Bacto® Tryptone</td>
<td>DIFCO, Augsburg</td>
</tr>
<tr>
<td>BCIP (5-bromo-4-chloro-3-indolyl phosphate)</td>
<td>SIGMA, Deisenhofen</td>
</tr>
<tr>
<td>BHI agar</td>
<td>DIFCO, Augsburg</td>
</tr>
<tr>
<td>BHI broth</td>
<td>DIFCO, Augsburg</td>
</tr>
<tr>
<td>Bisacrylamide</td>
<td>SERVA, Heidelberg</td>
</tr>
<tr>
<td>Boric acid</td>
<td>SIGMA, Deisenhofen</td>
</tr>
<tr>
<td>Bovine defibrinated blood</td>
<td>WDT, Hoyerhagen</td>
</tr>
<tr>
<td>Bovine serum</td>
<td>WDT, Hoyerhagen</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>NEW ENGLAND BIOLABS, Schwalbach</td>
</tr>
<tr>
<td>Bromphenol blue</td>
<td>SIGMA, Deisenhofen</td>
</tr>
<tr>
<td>Calf serum, heat inactivated</td>
<td>PAA LABORATORIES GmbH, Köln</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>SIGMA, Deisenhofen</td>
</tr>
<tr>
<td>Chloroform</td>
<td>ROTH, Karlsruhe</td>
</tr>
<tr>
<td>Columbia agar</td>
<td>Oxoid, Wesel</td>
</tr>
</tbody>
</table>
Appendix

$\alpha^{32}$P-dCTP NEN, Boston, MA, U.S.A.

L-Cysteine hydrochloride monohydrate SIGMA, Deisenhofen

2′-deoxynucleotide 5′-triphosphate (dNTP) ROTH, Karlsruhe

di-sodium hydrogen phosphate SIGMA, Deisenhofen

Dimethylsulfoxide (DMSO) ROTH, Karlsruhe

Ethanol Elch-Apotheke, Hannover

Ethidium bromide SIGMA, Deisenhofen

Ethylene diamine tetraacetic acid (EDTA) SIGMA, Deisenhofen

Formamid SERVA, Heidelberg

Glycerine ROTH, Karlsruhe

Glyoxal SIGMA, Deisenhofen

Hydrochloric acid ROTH, Karlsruhe

IPTG (Isopropyl-β-D-thiogalactosid) ROTH, Karlsruhe

Isoamyl alcohol MERCK, Darmstadt

Isopropanol ROTH, Karlsruhe

Kanamycin SIGMA, Deisenhofen

Methanol ROTH, Karlsruhe

Mineral oil SIGMA, Deisenhofen

MOPS ROTH, Karlsruhe

Polyethylene glycol 6000 (PEG 6000) MERCK, Darmstadt

Phenol (Aqua- Roti®-Phenol, water equilibrated ROTH, Karlsruhe

Phenol (Roti®-Phenol, TE equilibrated) ROTH, Karlsruhe

Pheny!-methyl-sulfonyl-fluoride (PMSF) SERVA, Heidelberg

Potassium acetate SIGMA, Deisenhofen
Appendix

Resazurine EGA CHEMIE, Steinheim
Ribonucleotide 5'-triphosphate ROTH, Karlsruhe
Sucrose MERCK, Darmstadt
Sarcosyl (N-lauryl-sarcosine) SIGMA, Deisenhofen
L-Serine SIGMA, Deisenhofen
Sodium acetate SIGMA, Deisenhofen
Sodium chloride SIGMA, Deisenhofen
di Sodium hydrogen phosphate (Na₂HPO₄) ROTH, Karlsruhe
Tris (Hydroxymethyl aminomethane) SIGMA, Deisenhofen
Trypticase soy broth BBL BECTON DICKINSON, Cockeysville, USA
Tween 20 ROTH, Karlsruhe
Urea ROTH, Karlsruhe
X-Gal (5-bromo-4-chloro-3-indolyl-galactosid) SIGMA, Deisenhofen
Xylene cyanol SIGMA, Deisenhofen
Xylitol SIGMA, Deisenhofen
Yeast extract ROTH, Karlsruhe

8.2 Sequence of A. pleuropneumoniae hybB

```
GAAGGAGGATCACCATGGCTAAAGCTCGTCCTCTCGGCGGTCGTTTAATATCAATCCCCG
1 ---------+---------+---------+---------+---------+---------+ 60
CTTCCTCTAGTGTTACCGATTTCGAGCAGGAGAGCCGCCAGCAAATTATAGTTAGGGGC
MAKARPLGGRLISIPV -
TATTGGTATTCGCACCGCTTGCCGCTATTTGCGCTTTATTAATATTACGTCGTCTGATTT
61 ---------+---------+---------+---------+---------+---------+ 120
ATAACCATAGCGTGCGCAACCGCGATAAACCGAATACTTTATATTATAATGCGAGACACTAAA
LVFAPLAAIALALILLRRRLIF -
```
Appendix

TCGGTATCGGTTCCGTGACCGAACTGAACGGCGGTTATCCTTGGGGGTTATGGATCGGTT
121 ---------+---------+---------+---------+---------+---------+ 180
AGCCATAGCCAAGGCACTGACTGGCTTACGTGCCGAATAGGAAACCCCAATACCTAGCCAA

G I G S V T E L N G Y P W G L W I G F -

HaeIII |

TCGACTTATTGGTGCGGAACCGGAGTTGCGCTGCGGCTGCTGCTGCTGCTGCTGCTGCTGCTG
181 ---------+---------+---------+---------+---------+---------+ 240
AGCTGAATAACCGCCTTGCCCTAACCGGACGCCCCGGGCAACCGCAACTGCCCTGTGCCTTCA

D L L V G T G L A C G G W A L A W T V Y -

AGCTGCTTAAATAAGGGATATAACCCACCTCTCTATGTAATGCCTCCGATATTAGCCAGCTTTAT
241 ---------+---------+---------+---------+---------+---------+ 300
TACAGAAATTTTCTCCTTTTTCGATATGGGTAATCATGCAGGGGTAATATCGTGCAATA

V F N K G K Y H P L V R P A L A S L F -

TCGATATACCTTCTGCGGCTTCTATCCATCTACTGAGATAGTGGCGGATTGTGCTTAT
301 ---------+---------+---------+---------+---------+---------+ 360
AGCTATATGCCATCGCGCAATAGGTAGTGATAGCTATACCCAGCAATAACCGTAAACG

G Y T L G G L S I T I D M G R Y W H L P -

CTTATTATTATCTGCGGCAACATTCATACCTCTCTCTCTATGTAATGGAAACCCGCGCCTAT
361 ---------+---------+---------+---------+---------+---------+ 420
GAATAAAAAGTGCGCCTGGTGAAGTTAGTTGGAAGAAGGTAATAAAACTTTGCGGCAAT

Y F F M P G Q F N T S S V L F E T A A C -

GTATGACCGGTTTATATTGTGTGGATGTAGGCGACCCGCTATTATTAAAGGTAATGATTCT
421 ---------+---------+---------+---------+---------+---------+ 480
CATACGTGCAAAATATAAACCACACACTGGAATCTCAAGCGTGGCCAGTAAAATCCAATGA

M T V Y I C V V T L E F A P V I L G Y F -

TCGTTTTGAAAAATGTTGCGATAAGCTGAAATAAACACATGTGTTTTTGTGTTGCGTTACT
481 ---------+---------+---------+---------+---------+---------+ 540
AGCCAACTTTTTTACCAAGCTATTACTGACATTTATTATTGTAACAAAAGAATCAACGCAATC

G L K K W F D K L N K I M F F L V A L G -

GGCGTTTTATTACCAGTATGCAACAAAAATTCATGTTTTTCTTACTGTTGCGTTAG
541 ---------+---------+---------+---------+---------+---------+ 600
CGCGAAATAATGGCTACTACCGTGGTTAGGAGTTACCAGGAAAATTACTAACAACGTCCAG

A L L P M M H Q S S M G S L M I V A G H -
ATAAGTACAACCCTGTATGGCAGATACGGCTCTCTCAATCTCTCAGTTATTTACCG

K V H P V W Q S Y E M L P I F S L F T A

CATTTATTATGGGCTTCTCCATTTATTTTGGAGGATATTAGTCAAAGGGGATTAG

F I M G F S I I I I F E G S L V K A G L A

CGGAAAAGCCACCGGACGAAAGACTTTATTATCCACAGTTTAGCAGATACCGGAAATTTC

G K A P D E R H L F T R L A R V A A I L

TAATCGGATTTATTAATCGCTTTCCGAGAGTTAATTATACAGATATTACATT

I G L F I V V R F G E L I Y N D K L H Y

ACGTCTTACAGCGATTTCTATTACTATTAATGTGGTTAGAAAGTTCATTATGTCCC

V L Q G D F Y S L M F W L E V S L M S L

TACCTATTTTCCCATTTATCTGAGGACTAATTCTGAAAAGGGTACATTACGGAATTACG

P I F T L F L G D K F S D S R W L F I S

CGGCACCTTTGTATGGGCTGCGGCGGCGCTTTGATAATTTCCATCTATTATGT

A L C M I G G S A L W R M N Y S I I M F

TTGATCCGGGATCCAGATTACTCTTCGGTGCAGGAACCTATAATTTCATTCATCT

D P G M G Y D Y F P S A P E L L I S I G

GTATTTATTTCTATTGAAATTTCGGCTATATATATTTGTATGTAGTTATTTCCGTAATTAC

F I S I E I C A Y I L I V R L F P V L P
110

CAGTTATCCAAGAAGCAGCATCAAGAAAATAATATTCAATCCGGAGTGAGAGCATGACAAC

---------+---------+---------+---------+---------+---------+ 1200

GTCAATAGGTTTCTTGAGTTACTATTATTTATTAAATAGTTAGCCCTCACTCTGGTACTGTGG

VIQEAHQNENIQSGVRA*

---------+---------+---------+---------+---------+---------+ 1260

TAAAACACGTATTACTATCGACCTGTATCGGTATCGAAGTCATTTACGTATCGATTTGT

1201 ---------+---------+---------+---------+---------+---------+ 1260

ATTGGTAGATAATGATCTGGGCAATGCGCATAGCTTCCAGTAAATGCAATAGCTA

TGAAATCGAAGACCGAAAAGTCAACAACTGTCTGGATCATGATCCATGTGCGAGGAGCAT

1261 ---------+---------+---------+---------+---------+---------+ 1320

---------+---------+---------+---------+---------+---------+ 1320

ACTTTAGCTTCTGCTTTTCAGTGTTACGAAAGCAGTAGCTCAATGCTGTTAAAAAC

1261 ---------+---------+---------+---------+---------+---------+ 1320

---------+---------+---------+---------+---------+---------+ 1380

CTTTATTATATAATATCTGGACTTCTAGAGCGCTACGAACTCAACTCTGGTTAGACCC

1321 ---------+---------+---------+---------+---------+---------+ 1380

---------+---------+---------+---------+---------+---------+ 1380

CGGCCACACTGATGCTGATGCTGATGCTGATGCTGATGCTGATGCTGATGCTGATGCTGATGCTG

1381 ---------+---------+---------+---------+---------+---------+ 1440

---------+---------+---------+---------+---------+---------+ 1440

TGCGAAATCGACTCTGTAAAAATATGAAAAGGCGTTACCTGAGCCTAAAATGCAATAGCTA

1441 ---------+---------+---------+---------+---------+---------+ 1500

---------+---------+---------+---------+---------+---------+ 1500

ACGCTTTCCATGGCATTTACCGGTATTTATATAGCGCAGTTACACCTGACCCCACCTAATAATGAAG

1501 ---------+---------+---------+---------+---------+---------+ 1560

---------+---------+---------+---------+---------+---------+ 1560

CGTACTGGTAGCTACATGATGAAAAATAGTTGAAAGGGGCGTTAATTGACCCACCCCACCTAATAATGAAG

1561 ---------+---------+---------+---------+---------+---------+ 1573

---------+---------+---------+---------+---------+---------+ 1573

ACGCGAGCTTCCG

8.3 Sequence of *A. pleuropneumoniae* furC, furP and furO

AAGGAGGTAAATATGCCATGGCTTTAAAACGCAAATTCGTGTGTCTTGTGCTTTAC

1 ---------+---------+---------+---------+---------+---------+ 60

TTCCCTCAAATTATACCCGATGAAATGTCTATTGGCTTATAAGCAACAGAAACGTTTTAATG

61 ---------+---------+---------+---------+---------+---------+ 120

---------+---------+---------+---------+---------+---------+ 120

CATTTTGCTTGGTAGACATTGTGTCGCTGCCACCGGAAAGTAAATACGTGTGGATAGTTG

61 ---------+---------+---------+---------+---------+---------+ 120

---------+---------+---------+---------+---------+---------+ 120

GTAAGAGCGATGCAATGCGGAGAAAAATTGTTAATACGTGTGGATACGTTT
Appendix

CGGTACCTTACGGCTTTGGCTGGGCTTTGGTATTTATTTGGTTTTATTTGCCG
2101 ---------+---------+---------+---------+---------+---------+ 2160
GCCATGAAATGCCGAAACCACGCCTCAGAATCCCAAAAAAAAAAAAAGCCTAAACAAACCGGC

CATTTGCCGTAGGGTTTGCGGCGGCGGAGTACCAGTCCGGTTTTGAGGCCGCTTCCATCG
2161 ---------+---------+---------+---------+---------+---------+ 2220
GTAACGCAGTGGGCGGGGATGATTCTGGTATCTTGGTATAAGAGGGAGTGGAGTACTAGGC

CTTTAATTACGGCGGGCTTTATGGCTTTTGCTTTATGCTGGGCTTTACGCTTTGATGAT
2221 ---------+---------+---------+---------+---------+---------+ 2280
GAAATTAATGCCGCCCCGGAATACAGCAGAAACGGAATAATCCGGAAGTGGCCAAACCAATTTTA

CTAAATGGTAGCAATTCTACTATTATGTGTTATATCTCTGATTGCGATTGCCGATCGCAGTACG
2281 ---------+---------+---------+---------+---------+---------+ 2340
GATTACAATGCTAAAGGATAATAAAACACATATAGGACTAAAGCTAAGGCGACTTGTCGAC

ATTTCGGTGCAGTTTTTTTGATTTGCTTCGTACAAATAATTTAGTGGTATCCGCAGAT
2341 ---------+---------+---------+---------+---------+---------+ 2400
TAAAAGCCAGCTTTAAAACCCGGAATAGCCGACGCGCTTAGGCTTTATGCGCTGCTTAGGAC

AAAAACCGCTTTAGGGCTTGTTAGACGGCGAGTTAGGAAACTGATACTTGGC
2401 ---------+---------+---------+---------+---------+---------+ 2460
TTTTGGAGATTACGAGGCGAGTTTTTGAATAGGCTAAGGCTTTATGCGCTGCTTAGGAC

AAAAGGTTACGCCGGTATAATAAAAATTTACACATCATTATCACTCATAAAA
2461 ---------+---------+---------+---------+---------+---------+ 2520
TTTTTTTTTTATGCCGGTCTTTTAAATTTTTAAATGAGTTAATTGCTAAGTATGATATTT

fucA Start
AATTTAACGGAGTTCTCATTGAAATCGCAGAAGTTGGCTCGACAAATTTAGTACCTTGCC
2521 ---------+---------+---------+---------+---------+---------+ 2580
TTAATTTTCCTAATTCGGACTCTTTGAAAAATACGCTTTATGCGCTAGGAGG

MNRRRELARQIIDTCL-
TTGAAATGACGTCTGTTTTGAAACCAAGGGCAGGGCTAATATTTGCTTAGCTATACG
2581 ---------+---------+---------+---------+---------+---------+ 2640
AACTTATACTGTCAGGACAAAATTGGGTTTTCCCCTGGCCGTCATTGAAATACGCGCATG

EMTRLGLNQGTAGNVSVRYQ-
AAGACGGTATCAGTTTTCCACGGCTTTTCAGCCGAGGAGTATAGAATGAGCTTGGAGG
2641 ---------+---------+---------+---------+---------+---------+ 2700
TTCTGCCATACGACTATTGGGGCTGCCCATTAGCAGGATCTTGGGTTTTACTGCTCTGGGCT

DGMILITPTGTGYPEDSII-

114
CACCCGGTCAAGGCGAATACCTTGACCAGTTTTTTTATGGTTAAGTAAAAAATATTCTCT

5821 ---------+---------+---------+---------+---------+---------+ 5880
GTGGGCCAGTCGCTTATGGAACCTGGCCTAAATTTAACATTTTTCATTTTTTTAATAGGA

XhoI
ATTTAAATTAATAGATTAAATTTTATATAAATTGAGTGAATAAACCTCAGAGTTTTTG

5881 ---------+---------+---------+---------+---------+---------+ 5940
TAAATTTAATTATCTAAATTTAATTATATATTAACTCATATCCTTTTAATTGGAGCTCAAAACT

AATGCTATTATTTACAAAAACATAAAAAACTGTGGTTTTTTTATTTTTATTTTTATAGGAAC

5941 ---------+---------+---------+---------+---------+---------+ 6000
TTACGATAAAGTTTTTGGTTATTTTTGACCAAAAATAAAAAATAAAAAAAGAAAAATAATCATCTTG

TTATTTGGGCACTTTCGAAACAAAACGAAAATCCGAGCCTGTCGTTACGGTTAGCTGAT

6001 ---------+---------+---------+---------+---------+---------+ 6060
AAATAATACCCTGATAGAGCTTTGGTTTTTGTTCCTAGGCGAGCAGCTACACTCCAAATCCTCT

TCGTCGGTATTATTCCCGGTTATTATTCCGGCATTCCGTTAAAATGGGTTCGGAACATCCTT

6061 ---------+---------+---------+---------+---------+---------+ 6120
AGCAGCCATAAATACGGCCTAAATTTAAGTCTGTAAGCATTCTTTTACCCACGCTTGTAGGA

TCCCCACGCTAGCCGATCTGATTTTATTAATCTGGGTCTCCGCACACGGTTTTTAGAGG

6121 ---------+---------+---------+---------+---------+---------+ 6180
AGGGTGGCAGCTGGCTAGCTAAATAGGTTAGAAGCTACACTCCAAATCCTCT

CATTCAATTCAGGCTCTATTAGCGATGAACACGCGATTAGACAGCAATGCTCTGCTCGGAT

6181 ---------+---------+---------+---------+---------+---------+ 6240
GTAATTAAATCCGAGTTAATCGCTACTTTTGTCGCGTAATCTCAGTTACGAGACGACCACA

TAAACCCCTCCGGCCTTTTTTTTCACTGATATACATCGTGATACCGACCTTTTATACACCGGTG

6241 ---------+---------+---------+---------+---------+---------+ 6300
ATTGGGGAGGCCCAAAAGGATGACACTAAATGAGCCATAGCTGGGAATATTGTTCACAA

CATTTACTTTTTCGCGATACACATTTAATCTCAATCGCGCTAACACATATGATTTTCTCAT

6301 ---------+---------+---------+---------+---------+---------+ 6360
GTAAATGAAAACCCTAGCTGTTGTGAAATTGAGTTAGGCGCTATTGTGTATACAAAGAGTA

TAGGTGTTTGGATCGGTATTATCGCGGAAGATATTCTCCTGCTACCAAGAATTTTCTGTC

6361 ---------+---------+---------+---------+---------+---------+ 6420
ATCACAACCGCTAGCCAAATCGAATACCGCGCTTTCTCATAAGGGCCTTTTATTTTAAAGACCCG

AAGGTTATCGGTGGGTTATTATCGCAAAATATCTGTGTAACACTACATTACATTCCGGGCAT

6421 ---------+---------+---------+---------+---------+---------+ 6480
TTCCATAGCCACCCGAAATAGGGCTTTTATAGACACATGTGTGTAAATGTGAAGGCGTACG

121
Appendix

TAGGCTTAACACCGCGGTGGCCGAATGGGCTTTATACGAACATATTTCCGAATTAGTCG
6481  +---------+---------+---------+---------+---------+---------+ 6540
ATCCGAATTGCGCGCAAGCCGCTTTACCGGGAATATATGCTTTGATAAAAGGCTTTAATCAGC

GTCACATCTTCTGGTTCTGGACAAATTGAGGTAATTCGTGCTGATTTTGACTGTAACCGTATTA
6541  +---------+---------+---------+---------+---------+---------+ 6600
CAGTGTAGAAGACCAAGACCTGTTAACTCCATTAAGCAGCACCTAAATGCAATTTGACAAATT

CCAAAGAGCCGATGCGAATCCGCTTTAAATCAAGCTCATCGTTAATTTATCGGTTAAA
6601  +---------+---------+---------+---------+---------+---------+ 6660
GGTTTCTCGCCTACGCCTTACCGGCAATTTAGTTCGAGTAGCAATTAAATAGCCAATTT

TGCAAAAAACACTCTTATCGAGTGTGTTTTTTTTTGGTTGCAAGCGGTCGAATTTTCCGTT
6661  +---------+---------+---------+---------+---------+---------+ 6720
ACGTTTTTTGAGAGATAGCTCACAAAAAAAGAAAACCCCAGTTCGCCAGCCTTAAAGCCGA

TTTCTTACCGTACAAAAATACCTAAACGCTATAAAAAAGCTTTTTTACTTTCCCTTTTC
6721  +---------+---------+---------+---------+---------+---------+ 6780
AAAGAATGGACATGTTTTATTGATTTGCGATATTTTTTCGAAGAATATGAAAGGAAAG

TAATTAATTTAATCTAAGCTTACCGGAGTTCCCTAGCTAGCTAGCCATGGC
6781  +---------+---------+---------+---------+---------+---------+ 6829
ATTATAATTTAATCTAAGCTTACCGGAGTTCCTAGCTAGCTAGCCATGGC

122
## 8.4 Animal experiment

<table>
<thead>
<tr>
<th>Reisolation from organ samples</th>
<th>BALF day 21</th>
<th>BALF day 7</th>
<th>ELISA ApxIIA/ detergent wash</th>
<th>lung score</th>
<th>clinical day 1</th>
<th>ear tag</th>
<th>group</th>
</tr>
</thead>
<tbody>
<tr>
<td>heart</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>day 1</td>
<td>6579</td>
<td>Appwt</td>
</tr>
<tr>
<td>lymph node</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>day 1</td>
<td>6593</td>
<td></td>
</tr>
<tr>
<td>lung altered</td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6575</td>
<td></td>
</tr>
<tr>
<td>lung unaltered</td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6696</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6706</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 2</td>
<td>6709</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6713</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6716</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6576</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6590</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6696</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6702</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6703</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6681</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6687</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6692</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6702</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6703</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6681</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6687</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6692</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6702</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6703</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6681</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6687</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6692</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6702</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>day 1</td>
<td>6703</td>
<td></td>
</tr>
</tbody>
</table>

Table 7: Raw data for challenge experiment with *A. pleuropneumoniae* and *A. pleuropneumoniae* ∆hyb
Table 8: Raw data for challenge experiment with *A. pleuropneumoniae* and *A. pleuropneumoniae Δfuo*

<table>
<thead>
<tr>
<th>group</th>
<th>ear tag</th>
<th>lung score</th>
<th>ELISA ApxIIA/detergent wash</th>
<th>Reisolation from organ samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>lung altered</td>
</tr>
<tr>
<td>Appwt</td>
<td>6757</td>
<td>23.6</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>6777</td>
<td>5.79</td>
<td>41/1:6400</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>6789</td>
<td>1.32</td>
<td>32/1:6400</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>6723</td>
<td>0</td>
<td>1/1:1600</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6775</td>
<td>9.36</td>
<td>6/1:12800</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6796</td>
<td>1.05</td>
<td>2/1:3200</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6688</td>
<td>4.76</td>
<td>16/1:6400</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6814</td>
<td>12.14</td>
<td>34/1:12800</td>
<td>+++</td>
</tr>
<tr>
<td>AppΔfuo</td>
<td>6699</td>
<td>9.82</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6704</td>
<td>5</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>6724</td>
<td>10.97</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>6776</td>
<td>0</td>
<td>3/1:1600</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>6675</td>
<td>5.79</td>
<td>15/1:51200</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>6813</td>
<td>8.39</td>
<td>9/1:12800</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>6691</td>
<td>5.55</td>
<td>10/1:6400</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>6798</td>
<td>5</td>
<td>26/1:12800</td>
<td>+++</td>
</tr>
</tbody>
</table>
8.5 Index of figures

Fig. 1: Pathways of aerobic and anerobic dissimilation of L-fucose by *E. coli* .................. 25
Fig. 2: Construction scheme and physical map of pHYB603................................................ 59
Fig. 3: Analysis of *A. pleuropneumoniae* and *A. pleuropneumoniae Δhyb* ..................... 60
Fig. 4: Body temperature of animals .................................................................................. 63
Fig. 5: Lung lesion score of animals.................................................................................. 64
Fig. 6: Humoral immune response of pigs.......................................................................... 64
Fig. 7: Construction of pFUO604......................................................................................... 68
Fig. 8: Analysis of *A. pleuropneumoniae* and *A. pleuropneumoniae Δfuo* .................... 70
Fig. 9: Body temperature of animals.................................................................................. 73
Fig. 10: Lung lesion score of animals................................................................................ 74
Fig. 11: Humoral immune response of pigs...................................................................... 74

8.6 Index of tables

Table 1: List of bacterial strains used in this study ................................................................. 28
Table 2: Plasmids used in this study ..................................................................................... 31
Table 3: Component in the PCR reaction............................................................................. 40
Table 4: Primers used in this study...................................................................................... 41
Table 5: Virulence study of *A. pleuropneumoniae* and *A. pleuropneumoniae Δhyb* ....... 65
Table 6: Virulence study of *A. pleuropneumoniae* and *A. pleuropneumoniae Δfuo* ........ 75
Table 7: Raw data for challenge experiment with *A. pleuropneumoniae* and
*A. pleuropneumoniae Δhyb* .............................................................................................. 123
Table 8: Raw data for challenge experiment with *A. pleuropneumoniae* and
*A. pleuropneumoniae Δfuo* .............................................................................................. 124
Acknowledgements

First and foremost, I would like to express my gratitude to my supervisor, Prof. Gerald-F. Gerlach, for giving me the opportunity to learn about this interesting work, fascinating molecular biology, for extensive advice and guidance.

I wish to thank all of the doctorands and members of „Institut für Mikrobiologie, Zentrum für Infektionsmedizin, Tierärztliche Hochschule Hannover“ for their friendship, friendly atmosphere in the laboratory and friendly support of my study, which they can do. I wish to thank Mr. Jörg Merkel for his consultation and helpfulness about computer to become good graphics.

I want to extend my special thanks to Mrs. Ledwoch for her attention to us, students from abroad.

I also want to thank the DAAD for financial support.

I wish to express my gratitude to my best friend, Khin, for moral support. And last but not least I also thank to my parents, who gave me all available support and encouragement since my childhood.