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1 **Selective Capture of Transcribed Sequences (SCOTS) of *Actinobacillus***
2 ***pleuropneumoniae* in the chronic stage of disease reveals an HlyX-regulated**
3 **autotransporter protein.**

4

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10 running title: **SCOTS** reveals HlyX-regulated autotransporter of *Actinobacillus*
11 *pleuropneumoniae*

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1 **ABSTRACT**

2 *Actinobacillus (A.) pleuropneumoniae*, an important respiratory pathogen in swine, is able to
3 persist in host tissues for extended periods of time. In the study presented here, Selective
4 Capture of Transcribed Sequences (SCOTS) analysis was used to identify genes expressed by
5 *A. pleuropneumoniae* in the chronic stage of the disease (21 days post infection). After
6 isolation and reverse transcription of RNA from infected lungs as well as from culture-grown
7 *A. pleuropneumoniae*, transcribed *A. pleuropneumoniae* sequences were captured from
8 infected lung tissue and subjected to a subtractive hybridization procedure of lung-derived
9 against culture-derived *A. pleuropneumoniae* cDNA. 29 of the 36 genes that were identified
10 as in vivo-expressed are involved in transport or metabolic processes. We identified a surface-
11 associated putative 104 kD subtilisin-like autotransporter serine protease, designated AasP,
12 which has not been described in *A. pleuropneumoniae* to date. The gene was shown to be
13 present in all 15 *A. pleuropneumoniae* serotypes. It is transcribed in porcine lung tissue on
14 days 7 and 21 post infection. Under anaerobic conditions in vitro, its expression depends on
15 the global anaerobic regulator HlyX. To our knowledge, this is the first report of an
16 autotransporter protein being regulated by a global anaerobic regulator.

1 INTRODUCTION

2 The ability of *Actinobacillus (A.) pleuropneumoniae* to persist in host tissues presents a major
3 challenge for the establishment of herds that are free of the pathogen (Fenwick and Henry,
4 1994, Haesebrouck et al., 1997, Chiers et al., 1998). The adaptive mechanisms employed by
5 *A. pleuropneumoniae* to ensure long term survival in the host are largely unknown to date.
6 While genes that are upregulated in *A. pleuropneumoniae* in vivo have been the target of
7 recent studies, so far these studies have focused on the acute stage of the disease (Fuller et al.,
8 1999, Fuller et al., 2000, Sheehan et al., 2003, Baltes and Gerlach, 2004).
9 Selective capture of transcribed sequences (SCOTS) analysis has proved itself to be a useful
10 tool in studying bacterial gene expression directly in infected tissue, and has led to the
11 identification of hitherto undescribed autotransporter adhesins on day 7 post infection in *A.*
12 *pleuropneumoniae* (Baltes and Gerlach, 2004).
13 Autotransporter proteins associated with virulence have been identified in a number of
14 gramnegative bacteria. All of them share a common design in their protein sequence,
15 consisting of an amino-terminal signal sequence required for Sec-dependent transport through
16 the inner membrane, followed by the secreted portion of the protein termed the “passenger
17 domain”, and finally, the “translocator domain” which inserts into the outer membrane,
18 forming a pore to mediate the passage of the passenger domain to the outside of the cell
19 (Henderson et al., 2004). Autotransporter proteins can perform a diverse array of functions,
20 among them adhesion (Hia, Hsf in *Neisseria meningitidis*; St Geme J.W.III et al., 1996, Yeo
21 et al., 2004) or toxicity (Pet enterotoxin in *Escherichia coli*; Eslava et al., 1998).
22 Autotransporter proteins containing a serine protease motif are involved in the processing of
23 other autotransporters (SphB1 in *Bordetella pertussis*; Coutte et al., 2001) or cleavage of host
24 immunoglobulins (IgA1 protease in *Neisseria meningitidis*; Vidarsson et al., 2005), but can
25 also exhibit toxin characteristics, such as the secreted autotransporter toxin, Sat, of *E. coli*
26 (Guignot et al., 2006).

1 In the study presented here, we describe the use of SCOTS analysis for studies of *A.*
2 *pleuropneumoniae* gene expression in the chronic stage of *A. pleuropneumoniae* infection,
3 and the identification of a putative autotransporter serine protease which is expressed by *A.*
4 *pleuropneumoniae* in the porcine lung on day 21 post infection, and which is regulated by the
5 global anaerobic regulator HlyX.

6

7 **MATERIALS AND METHODS**

8 **Bacterial strains, plasmids, and primers.** The strains, plasmids, and primers used in this
9 work are listed in Table 1.

10 **Media and growth conditions.** *E. coli* strains were cultured in LB-medium supplemented
11 with ampicillin (100 µg/ml). *A. pleuropneumoniae* strains were routinely cultured in PPLO
12 medium (Difco GmbH, Augsburg, Germany) supplemented with nicotinamide dinucleotide
13 (NAD; 10 µg/ml; Merck, Darmstadt, Germany), L-glutamine (100 µg/ml; Serva, Heidelberg,
14 Germany), L-cysteine-hydrochloride (260 µg/ml; Sigma-Aldrich), L-cystine-dihydrochloride
15 (10 µg/ml; Sigma-Aldrich), Dextrose (1 mg/ml), and Tween[®] 80 (0.1%) and harvested at an
16 OD₆₀₀ of 0.4 or as indicated.

17 For anaerobic cultures, *A. pleuropneumoniae* from a fresh overnight culture was inoculated
18 1:10 in 50 ml of fresh medium and grown to an OD₆₀₀=0.3 before being inoculated 1:100 into
19 50 ml of PPLO medium that had been pre-incubated for 48 hours in an anaerobic chamber.
20 Anaerobic cultures were grown for 6 hours which corresponds to the late exponential phase.

21 **Manipulation of DNA.** DNA-modifying enzymes were purchased from New England
22 Biolabs (Bad Schwalbach, Germany) and used according to the manufacturer's instructions.
23 *Taq* polymerase was purchased from Gibco-BR Life Technologies (Karlsruhe, Germany).
24 Chromosomal DNA for PCR and Southern Blotting as well as plasmid DNA was prepared by
25 standard protocols (Sambrook et al., 1989). PCR, Southern Blotting, transformation and gel
26 electrophoresis were done by standard procedures (Sambrook et al., 1989).

1 **Animal experiments**

2 Tissue samples were obtained from five pigs from the wild type-infected control group of a
3 study investigating an *A. pleuropneumoniae* aspartase mutant (Jacobsen et al., 2005). The
4 animals had been infected with wild type *A. pleuropneumoniae* AP76 in an aerosol model.

5 Tissue samples were taken from necrotic lung tissue sections and immediately immersed in
6 RNAlater[®] solution (Ambion Inc., Houston, Texas).

7 Pigs were cared for in accordance with the principles outlined in the *European Convention for*
8 *the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes*
9 (European Treaty Series, no. 123: <http://conventions.coe.int/treaty/EN/MenuPrincipal.htm>),
10 and the experiments were approved by an ethics committee.

11

12 **Selective Capture of Transcribed Sequences**

13 Selective Capture of Transcribed Sequences (SCOTS) was performed as described previously
14 (Baltes and Gerlach, 2004), using necrotic lung tissue samples collected at day 21 post *A.*

15 *pleuropneumoniae* infection containing at least 10⁶ bacteria per gram tissue. For RNA

16 isolation from tissues, 30 mg samples preserved in RNAlater[®] solution (Applied Biosciences,
17 Darmstadt, Germany) were used. Culture samples (6 ml OD₆₀₀=0.4) were chilled on ice and

18 centrifuged for 5 min at 7,000 x g to pellet bacteria. RNA was isolated using the FastRNA[®]

19 Blue Kit (QBiogene, Heidelberg, Germany), using A FastPrep[®] instrument (QBiogene). For

20 tissue samples, lysing matrix E (QBiogene) was used instead of the matrix supplied with the

21 FastRNA Blue kit. RNA integrity was verified by visualization on an agarose gel. For use in

22 subsequent experiments, RNA from five animals was pooled at equal proportions. RNA

23 samples were treated with TurboDNase (Applied Biosciences, Darmstadt, Germany)

24 according to the manufacturer's instructions, and absence of genomic DNA was confirmed by

25 PCR using primers oRN5-1 and oRN5-2 which amplify a 180 bp fragment of the DMSO

26 reductase gene (Baltes et al., 2003). For subtraction, *A. pleuropneumoniae* AP76 RNA was

1 prepared from liquid cultures that had been grown aerobically with shaking to an OD₆₀₀ of
2 0.4. Lung-derived as well as *A. pleuropneumoniae* culture-derived RNA were subjected to
3 reverse transcription using primers oSCOTS-N9-1 or oSCOTS-N9-2 consisting of a defined
4 terminal sequence at the 5' end and a random 9mer at the 3' end (Table 1), according to the
5 descriptions of Daigle et al. (2001). Each cDNA population was subjected to three rounds of
6 normalization to reduce sequences transcribed in abundance (rRNA sequences in particular)
7 which would otherwise lead to false positives and/or loss of rare transcripts. One round of
8 normalization consists of hybridization of cDNA to biotinylated genomic *A.*
9 *pleuropneumoniae* AP76 DNA which has been preblocked with PCR products representing
10 16S and 23S *A. pleuropneumoniae* rRNA sequences (Table 1). For subsequent enrichment of
11 sequences preferentially transcribed or upregulated during growth in porcine lung tissue,
12 biotinylated genomic *A. pleuropneumoniae* AP76 DNA was then preblocked with rRNA PCR
13 products and culture-derived cDNA after three rounds of normalization. Three rounds of
14 capture hybridization with normalized lung-derived cDNA were then performed. Lung-
15 specific cDNA fragments were cloned using the TOPO[®] TA Cloning kit (Invitrogen,
16 Karlsruhe, Germany) and transformed into *E. coli* Top10 F' cells according to the
17 manufacturer's instructions. Cloned inserts were amplified by PCR using M13 forward and
18 reverse primers (Amersham Biosciences), spotted on nylon membranes, and subjected to
19 Southern dot blot analysis using ³²P-dCTP-labeled lung and culture specific cDNA pools
20 obtained after three rounds of normalization, respectively. Dot blots were washed with 3 x
21 SSC, 0.1% SDS at 60°C. In order to allow comparison of signal intensities in both blots, 1
22 and 0.1 µg of genomic *A. pleuropneumoniae* AP76 DNA spotted on nylon membranes were
23 used as positive controls and exposed alongside their corresponding SCOTS clone dot blots.
24 Clones showing weaker or no hybridization to the culture specific probe when compared to
25 the lung specific probe were analysed by nucleotide sequencing in a commercial laboratory
26 (SeqLab, Goettingen, Germany).

1 **Sequence analyses**

2 Sequence analyses were carried out using BLAST (Altschul et al., 1990) and MOTIFS
3 algorithms available in the HUSAR program package at the Deutsches
4 Krebsforschungszentrum (dkfz) in Heidelberg, Germany. Sequence data were also compared
5 to the database of the unfinished genomic sequence of *A. pleuropneumoniae* in a BLAST
6 search available at http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi. Promoter prediction
7 was performed using the Virtual Footprint promoter analysis tool (Munch et al., 2005)
8 available at <http://prodoric.tu-bs.de/vfp/>). Secondary structure predictions were carried out
9 using the PSIPRED structure prediction server (McGuffin et al., 2000,
10 <http://bioinf.cs.ucl.ac.uk/psipred/>).

11 **RT-PCR**

12 Aerobic *A. pleuropneumoniae* cultures were grown in a shaking incubator to $OD_{600} = 0.2, 0.4,$
13 $0.6, 0.8$ or 1.3 .

14 For RT-PCR, RNA was prepared and DNase treated as described above, and reverse
15 transcription was carried out using 5 μ g of RNA in the presence of 5 pmol of *aasP*-specific
16 reverse primers oSER2 and primer oRRN16-6 (internal positive control for 16S RNA gene)
17 together in the same reaction. Control reactions were set up without reverse transcriptase. The
18 cDNA template was then diluted 1:100 with ddH₂O for use in subsequent PCR. 30 cycles of
19 PCR were performed using primer pairs oSER1/oSER2 or oRRN16-3/oRRN16-6 in separate
20 vials, but from the same master mix, using 5 μ l of diluted cDNA as template. RT-PCR
21 experiments were performed at least three times.

22 **Harvesting of anaerobic cultures and preparation of bacterial membranes for SDS**

23 **PAGE, and Q-TOF mass spectrometry**

24 Cells were harvested by centrifugation (13,000 x g, 10 min, 4°C), washed once with a buffer
25 containing 10 mM Tris-HCl (pH 8.0) and 5 mM magnesium acetate. Pellets were resuspended
26 in 50 mM Tris-HCl (pH 7.3) and stored at -70°C overnight. Cells were thawed and then

1 ruptured using the FastPrep® Instrument (Qbiogene, Heidelberg, Germany) three times for 40
2 sec at intensity setting 5.0. Unbroken cells were removed by centrifugation (15,000 x g, 30
3 min).

4 The preparation of outer membranes was performed as previously described (Bolin et al.,
5 1982) with the following modifications: after ultracentrifugation of disrupted cells in an
6 SW55 Ti rotor at 45,000 rpm for 2 hours, the pellet containing the membranes was
7 resuspended in SM buffer (1 % sarkosyl, 1 mM β -mercaptoethanol) and incubated overnight
8 at 4°C. After a second round of ultracentrifugation, the pellet contained integral membrane
9 proteins as well as hydrophobic proteins and was resuspended in a buffer containing 30 mM
10 Tris pH 8.5, 2M thiourea, 7 M urea and 4 % CHAPS. Membrane proteins were separated on a
11 10.8 % SDS polyacrylamide gel at 15 mA for 12 hours.

12 Proteins in analytical gels were visualized by staining the gels with silver nitrate (Sigma-
13 Aldrich, Munich, Germany), preparative gels were stained with colloidal Coomassie G-250
14 (Candiano et al., 2004).

15 For Q-TOF analysis, protein bands were cut from a Coomassie-stained gel; the protein was
16 trypsinated and extracted from the gel by using a slightly modified method by Wilm and
17 Mann (Shevchenko et al., 1996). Briefly, the gel pieces were dehydrated with acetonitril
18 (Merck, Darmstadt, Germany), rehydrated with a 100 mM NH_4HCO_3 buffer containing 10
19 mM DTT (Roth, Karlsruhe, Germany), and then treated with 100 mM iodoacetamide (Sigma-
20 Aldrich) in 100 mM NH_4HCO_3 . Dehydration and rehydration were repeated, and dehydrated
21 gel pieces were rehydrated with buffer (50 mM NH_4HCO_3) containing 20 ng/ μl trypsin
22 (sequencing grade; Promega, Mannheim, Germany) and incubated for 12-16 h at 37°C.

23 Peptides were extracted using 50 mM NH_4HCO_3 followed by extraction buffer containing
24 50% (vol/vol) acetonitril (Merck) and 5% (vol/vol) formic acid (Merck). The solution
25 containing the extracted peptides was completely evaporated in a vacuum centrifuge. Peptides
26 were resuspended in 50% (vol/vol) acetonitril (Merck), 0.1% (vol/vol) formic acid (Merck).

1 Peptide sequences were determined from tandem mass spectrometry (MS/MS) fragmentation
2 data recorded on an electrospray injection quadrupole time of flight mass spectrometer (ESI
3 Q-ToF MS; Q-Tof Ultima, Waters, Milford, Massachusetts, USA). Proteins were identified
4 using the program ProteinLynx Globals Server (Version 2.1, Waters) by searching against the
5 National Centre for Biotechnology Information (NCBI) complete database
6 (<ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/>).

7

8 **RESULTS**

9 **SCOTS analysis from day 21 post infection**

10 Of 74 sequenced clones, 16 were found to contain rRNA sequences. The remaining 58 clones
11 contained 36 unique genes which are listed in Table 2. They can be divided into five
12 functional groups: cell surface, metabolism, regulatory, stress, and transport (Table 2).

13 The regulatory proteins identified were HlyX (clone 1E9), a global anaerobic regulator of *A.*
14 *pleuropneumoniae* resembling FNR of *E. coli* (Soltes and MacInnes, 1994) as well as two
15 proteins involved in transcription termination and antitermination, the Rho protein (clone
16 2B2) and NusA (clone 1A8)(Ciampi, 2006).

17 Among the transport proteins, general transport protein SecA was identified (clone 1A1), as
18 well as components of several amino acid or peptide ABC transporters (clones 1H3, 1E11,
19 2C5, 2D8). One protein involved in sugar transport, a xylose binding protein (clone 1C4), was
20 found. One identified protein is possibly involved in mercury ion transport (clone 1F11). For
21 one transport-related protein, no possible substrate could be identified (clone 1A7).

22 Three stress-related proteins were identified, namely a DNA or RNA helicase (clone 1B1),
23 elongation factor Tu (clone 1B8), and DnaK (clone 1D4).

24 The group of metabolic genes identified contains proteins involved in cell respiration and
25 production of energy such as ubiquinone reductase (clone 2G4) and ATP synthase (clone
26 1C12), and also proteins involved in nucleic acid and protein synthesis, like DNA primase
27 (clone 1A10), RNA polymerase B (clone 1G3), and ribosomal proteins (clones 1H7 and 1D1).

28 Several proteins involved in the synthesis of cell components were identified, such as
29 dihydropteroate synthase (clone 1G10), MreB (1G6) and n-acetylmannosamine-6-phosphate-
30 epimerase (1D9). Three antibiotic-modifying enzymes (for streptomycin, beta lactam

1 antibiotics, and tetracycline; clones 1A6, 2A6, and 2C7, respectively) were identified in this
2 group.

3 Three of the genes identified in this study were also found in a previous SCOTS analysis of *A.*
4 *pleuropneumoniae* investigating gene expression on day 7 after infection, namely EF-Tu
5 (clone 1B8), ubiquinone reductase (clone 2G4), and RNA polymerase B (clone 1G3).

6 A single cell surface associated gene was identified (clone 2E12), encoding a putative serine
7 protease autotransporter protein termed AasP. Since a serine protease autotransporter has not
8 been described in *A. pleuropneumoniae* to date, and since in other gramnegative bacteria,
9 autotransporter proteins have been implicated in pathogenesis (Henderson and Nataro, 2001),
10 this gene was chosen for more detailed analysis.

11

12 **Putative autotransporter serine protease AasP**

13 **Sequence analyses.** The only gene predicted to encode a surface-associated protein that was
14 identified in this work was the putative subtilisin-like autotransporter serine protease gene
15 *aasP*, present in two sequenced clones. The fragment identified in SCOTS analysis was used
16 to search genomic data of *A. pleuropneumoniae* serotype 1. The obtained match
17 (ZP_00135639) was used to derive PCR primers for amplification and subsequent nucleotide
18 sequencing of the gene in *A. pleuropneumoniae* serotype 7, which was identical to the *A.*
19 *pleuropneumoniae* serotype 1 gene and designated *aasP* (GenBank accession no. DQ490067).
20 Using primers oSER1 and oSER2 in PCR analyses, the *aasP* gene could be detected in all *A.*
21 *pleuropneumoniae* reference strains (not shown).

22 The putative promoter region of *aasP* was found to contain a Shine-Dalgarno consensus
23 sequence (AGGAGA, centered at -10; Fig. 1). Using the Virtual Footprint promoter analysis
24 tool (Munch et al., 2005) to look for anaerobic regulator FNR binding sites up to 800 bp
25 upstream of the start codon, a single putative binding site for the FNR homolog HlyX was
26 identified. Its sequence is TTGAG---ATCAA and it is centered at position -78. It shows two
27 mismatches in comparison to the HlyX binding site of the *A. pleuropneumoniae*
28 dimethylsulfoxide reductase (TTGAT-----ATCAG, (Baltes et al., 2003) but only a single

1 mismatch in comparison to the proposed *E. coli* consensus sequence for FNR binding
2 (TTGAT----ATCAA, (Guest et al., 1996, Fig. 1).

3 The 2799 bp *aasP* open reading frame (ORF) encodes a protein of 932 amino acids in length
4 (104 kDa) which is 58% identical to Ssa1, a surface antigen of *Mannheimia haemolytica* (Lo
5 et al., 1991). Other similar proteins are SphB1 of *Bordetella pertussis* (24% identity, 43%
6 similarity; (Coutte et al., 2001) and AusP of *Neisseria meningitidis* (32% identity, 43%
7 similarity; (Turner et al., 2002). Using the SignalP Server, a predicted signal peptide cleavage
8 site was identified between Ala²⁷ and Asp²⁸.

9 The PsiPred secondary structure prediction algorithm revealed a beta domain starting at His⁶⁶⁴
10 and ending with Phe⁹³², resulting in a 31 kDa domain. The predicted beta domain is preceded
11 by a putative linker region, which is an alpha-helical domain spanning 49 amino acids (Tyr⁶¹⁴-
12 Glu⁶⁶³). These findings are consistent with those for other autotransporter proteins, in which
13 the beta domain and linker region constitute the translocation unit (Maurer et al., 1999, Oliver
14 et al., 2003)

15 Similar to the subtilisin-like autotransporter serine proteases from *M. haemolytica* Ssa1, (Lo
16 et al., 1991), the SphB1 protein from *B. pertussis* (Coutte et al., 2001), and the AusP protein
17 from *N. meningitidis* (Turner et al., 2002), the *A. pleuropneumoniae* AasP protein sequence
18 contains a subtilase motif (the active site for proteolytic activity), spanning from Gly³⁴⁹ to
19 Gly³⁵⁹ (Fig. 2).

20 **Analysis of transcription.** Transcription of *aasP* in vitro and in vivo was investigated by RT-
21 PCR analysis. Since *A. pleuropneumoniae* RNA content could not be determined in the
22 mixture of porcine and bacterial RNA obtained from the lung at day 21 post infection, reverse
23 transcription was carried out using 5 µg of total RNA from infected porcine lung tissue and
24 cultured bacteria, respectively. After 30 cycles of PCR, a serine protease product was
25 amplified from the 1:100 dilution of lung-derived cDNA, while culture-derived cDNA
26 yielded no product (Fig 3). The PCR product was verified by nucleotide sequencing.

1 In order to determine whether *aasP* transcription is dependent on oxygen levels and regulated
2 by the global anaerobic regulator HlyX, an RT-PCR experiment was set up using cDNA from
3 aerobic cultures of *A. pleuropneumoniae* AP76 harvested at $OD_{600} = 0.2, 0.4, 0.6, 0.8$ and 1.3 ,
4 respectively, as well as from anaerobically grown *A. pleuropneumoniae* AP76 and from the
5 isogenic HlyX deletion mutant *A. pleuropneumoniae* $\Delta hlyX$. In aerobic culture samples, the
6 transcript was not consistently detected every time the PCR was run. In anaerobic *A.*
7 *pleuropneumoniae* AP76 samples, an *aasP* transcript was always detected after 30 cycles of
8 PCR, while it was always absent in the *hlyX* deletion mutant. 16S RNA transcripts were
9 consistently detected in all samples. (Fig. 3).

10 **Analysis of protein expression.** AasP protein expression and subcellular localization was
11 examined by SDS PAGE. A prominent band approximately 100 kDa in size was detected in
12 membrane preparations of *A. pleuropneumoniae* AP76 wild type grown under aerobic and
13 anaerobic conditions, and in the HlyX negative mutant *A. pleuropneumoniae* $\Delta hlyX$ under
14 aerobic conditions, but was absent in an HlyX negative mutant under anaerobic conditions
15 (Fig. 4). The bands were excised from a Coomassie Blue stained gel and analyzed by Q-TOF
16 mass spectrometry. A peptide mass fingerprint was calculated and entered in a database
17 search using the MASCOT server (available at <http://www.matrixscience.com/server.html>),
18 which identified the protein as AasP with 35% sequence coverage. In MS/MS mode, the
19 protein was identified as AasP by de-novo sequencing with 4.6 % (aerobic) and 5.7 %
20 (anaerobic) sequence coverage. In aerobic membrane preparations, the three individual
21 peptides sequenced via Q-TOF MS/MS were N²⁶²-K²⁷⁴, F⁷⁰⁵-R⁷¹⁷ (oxidized form) and F⁸⁴⁷-
22 R⁸⁶³. In the anaerobic cultures, a total of five peptides were sequenced. Two of these peptides
23 were also identified in aerobic cultures, namely F⁷⁰⁵-R⁷¹⁷ and F⁸⁴⁷-R⁸⁶³; additionally, an
24 oxidized form of F⁷⁰⁵-R⁷¹⁷ was identified, along with peptides D³⁷⁸-K³⁹⁰ and Y³⁹⁶-K⁴⁰⁵.

25

1 DISCUSSION

2 One way to elucidate the mechanisms responsible for persistence of *A. pleuropneumoniae* is
3 the identification of genes that are expressed by the pathogen in the host in the later stages of
4 the disease. The analysis of gene expression directly in host tissues should be a favoured
5 approach. Using SCOTS analysis to compare *A. pleuropneumoniae* gene expression in
6 necrotic lung tissue on day 21 post infection and in liquid culture, we were able to identify 36
7 putative *in vivo* expressed *A. pleuropneumoniae* genes. Three of these genes, namely those
8 coding for elongation factor EF-Tu (clone 1B8), ubiquinone reductase (clone 2G4), and RNA
9 polymerase B (clone 1G3) have previously been identified as *in vivo* expressed in a SCOTS
10 analysis using tissue from day 7 post *A. pleuropneumoniae* infection (Baltes and Gerlach,
11 2004). Ubiquinone reductase (clone 2G4) has also previously been identified as essential *in vivo*
12 for *A. pleuropneumoniae* through STM analysis, along with DnaK (clone 1D4) (Fuller et
13 al., 2000, Sheehan et al., 2003). A dihydropteroate synthase (clone 1G10) has been previously
14 identified by SCOTS analysis as *in vivo* expressed in avian pathogenic *E. coli* (Dozois et al.,
15 2003).

16 The global anaerobic regulator HlyX (MacInnes et al., 1990) was identified in our analysis.
17 This is not surprising, as we can safely assume the inside of necrotic lung lesions to be
18 deprived of oxygen due to a lack of blood supply, which would lead to an upregulation of
19 anaerobically regulated genes and therefore an increased need for the HlyX protein itself. The
20 HlyX negative *A. pleuropneumoniae* mutant *A. pleuropneumoniae* Δ *hlyX* has previously been
21 shown to be impaired in its survival in sequestered lung tissue (Baltes et al., 2005); suggesting
22 that the observations made in infection experiments using the HlyX mutant result from an
23 insufficient ability to adapt its metabolism and transport mechanisms to anaerobic conditions.

24 The majority of genes identified in this study play roles in either nutrient transport, energy
25 metabolism, or synthesis of nucleic acids or cell components. This finding likely reflects the
26 differences between culture of the microorganism in nutrient broth and growth of a pathogen

1 within the host, however, even though these genes may not appear to be virulence or
2 persistence associated at first glance, they may be involved indirectly by sustaining basic cell
3 functions. For example, xylose has recently been shown to be a main component of biofilms
4 in *Bordetella bronchiseptica* (Irie et al., 2006), and a xylose transporter as identified here may
5 contribute to biofilm formation in *A. pleuropneumoniae* as well.

6 The gene we deemed most likely to have a more direct influence on *A. pleuropneumoniae*
7 pathogenesis was the only surface-associated gene we identified, a putative subtilisin-like
8 autotransporter protease termed AasP (GenBank accession no. DQ490067). Surprisingly, its
9 promoter region contains a putative HlyX binding site. Autotransporter proteins are usually
10 involved in pathogenesis (Henderson and Nataro, 2001), and deletions in several HlyX
11 regulated genes as well as in HlyX itself have been shown to have an attenuating effect on *A.*
12 *pleuropneumoniae* (Baltes et al., 2003, Jacobsen et al., 2005, Baltes et al., 2005). Subtilisin-
13 like autotransporter proteases have not been described in *A. pleuropneumoniae* to date.

14 The AasP protein sequence contains a typical subtilase motif which constitutes a putative
15 active site, and AasP is very similar to a number of known bacterial autotransporter subtilases
16 such as SphB1 of *B. pertussis*, which serves as a maturation protease for other autotransporters
17 (Coutte et al., 2001).

18 Since we suspected that *aasP* transcription was controlled by HlyX under anaerobic
19 conditions, we investigated *aasP* transcription in various stages of growth, in lung tissue, and
20 under anaerobic conditions. Using RT-PCR, we could demonstrate that the *aasP* gene is
21 transcribed in necrotic lung tissue and under aerobic and anaerobic conditions *in vitro*, but it
22 is not transcribed in an anaerobically grown HlyX deletion mutant. The inconsistencies in
23 transcript detection under aerobic conditions may hint at low transcript levels leading to
24 occasional detection failure.

25 On the protein level, we could show that under aerobic conditions, the AasP protein is present
26 in membrane preparations of *A. pleuropneumoniae* AP76 wild type and the *A.*

1 *pleuropneumoniae* $\Delta hlyX$ mutant. However, under anaerobic conditions, AasP is present only
2 in the *A. pleuropneumoniae* wild type and absent from the $\Delta hlyX$ mutant. These findings
3 confirm that expression of *aasP* transcription is HlyX dependent under anaerobic conditions.
4 The size of the identified protein shows that significant amounts of the protein remain
5 membrane associated. Under aerobic conditions, the protein expression levels do not
6 correspond well with the RT-PCR findings, therefore the regulation of AasP under aerobic
7 conditions remains to be investigated. The role of other regulatory proteins needs to be
8 examined; additionally, it is possible that posttranscriptional regulation rather than regulation
9 of transcript numbers plays a role in AasP expression under aerobic conditions.
10 As we show, *aasP* transcription does not occur in lung tissue exclusively (as the rationale
11 behind the SCOTS technique would suggest), but also under standard culturing conditions.
12 The same can be assumed for several other genes picked up in this SCOTS analysis, such as
13 HlyX, EF-Tu, and DnaK. These are most likely not only expressed *in vivo*, but are present
14 under standard culturing conditions as well. This can be explained by an *in vivo* increase in
15 transcription, leading to an abundance of transcripts in the *in vivo* cDNA pool, which could in
16 turn lead to incomplete removal of such transcripts from the cDNA pool during subtractive
17 hybridization, in spite of the excess of *in vitro* transcripts used for pre-blocking of
18 biotinylated DNA (Dozois et al., 2003). While such genes may still play a role for *A.*
19 *pleuropneumoniae* survival in sequestered lung tissue, *in vivo* expression and significance of
20 the genes identified in this study would have to be verified for each gene individually.
21 Likewise, the actual function of AasP remains to be determined, and it should be investigated
22 whether all of the protein remains membrane associated or whether it can also be cleaved to
23 release the passenger domain. The similarity of AasP to other autotransporter serine proteases
24 suggests a role in the processing of other autotransporters, namely adhesins, and thus, a
25 possible role in colonization, biofilm formation or persistence by *A. pleuropneumoniae*.
26 However, while other autotransporter proteins have been identified in *A. pleuropneumoniae*,

1 they have not yet been characterized. Therefore, their role as possible substrates for AasP is
2 unknown.

3 To our knowledge, this is the first report of an autotransporter protein whose transcription is
4 controlled by a global anaerobic regulator.

5

6

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11 DFG.

1 **FIGURE LEGENDS**

2 **Fig. 1: Promoter region of *A. pleuropneumoniae* *aasP*.** SD indicates the position of the
3 Shine-Dalgarno consensus sequence.

4

5 **Fig. 2: CLUSTAL alignment of autotransporter serine proteases of *A. pleuropneumoniae***
6 **(*AasP*), *Mannheimia haemolytica* (*Ssa1*), *Bordetella pertussis* (*SphB1*), and *Neisseria***
7 ***meningitidis* (*AusP*).** Asterisks mark the subtilase motif.

8

9 **Fig. 3: RT-PCR analysis of *aasP* transcription.** *aasP*: primers oSER1 and oSER2 for
10 amplification of serine protease gene fragment; *rrn*: primers oRRN16-3 and oRRN16-6 for
11 amplification of 16S rRNA gene fragment; cDNA templates: 1:100 dilutions. +: positive
12 controls for oSER and oRRN primers using AP76 DNA as template; **A:** transcription of *aasP*
13 under standard culturing conditions at OD₆₀₀=0.4 (lane 1) as well as in lung tissue at days 7
14 and 21 post infection (lanes 2 and 3, respectively); **B:** transcription of *aasP* in vitro at
15 different stages of growth: aerobically grown *A. pleuropneumoniae* AP76 at OD₆₀₀=0.2 (lane
16 1), 0.4 (lane 2), 0.6 (lane 3), 0.8 (lane 4) and 1.3 (lane 5), anaerobically grown *A.*
17 *pleuropneumoniae* AP76 (lane 6), and anaerobically grown *A. pleuropneumoniae* Δ *hlyX* (lane
18 7).

19

20 **Fig. 4: Protein expression analysis of *A. pleuropneumoniae* AasP in late exponential**
21 **phase.** Silver stained SDS-PAGE gel of membrane preparations of *A. pleuropneumoniae*
22 AP76 (wt) and *A. pleuropneumoniae* Δ *hlyX* (Δ) under aerobic (left) and anaerobic (right)
23 conditions. Arrows indicate bands identified as AasP by Q-TOF MS analysis.

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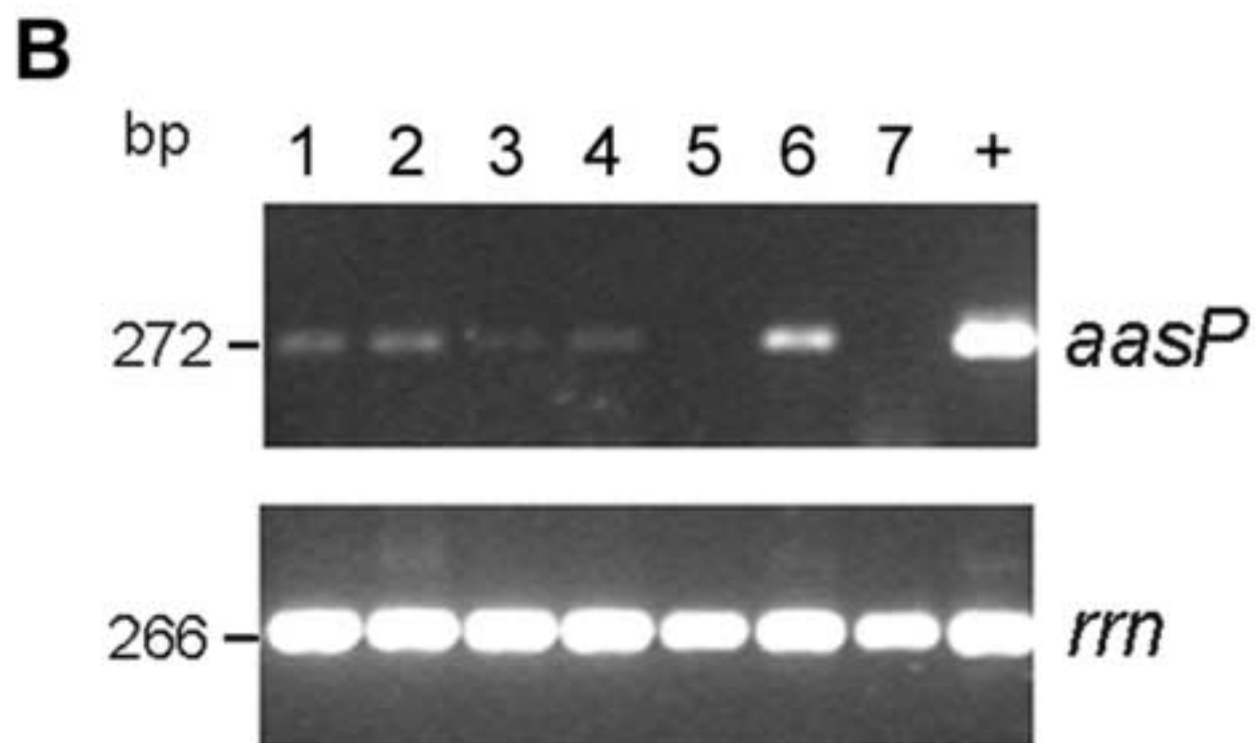
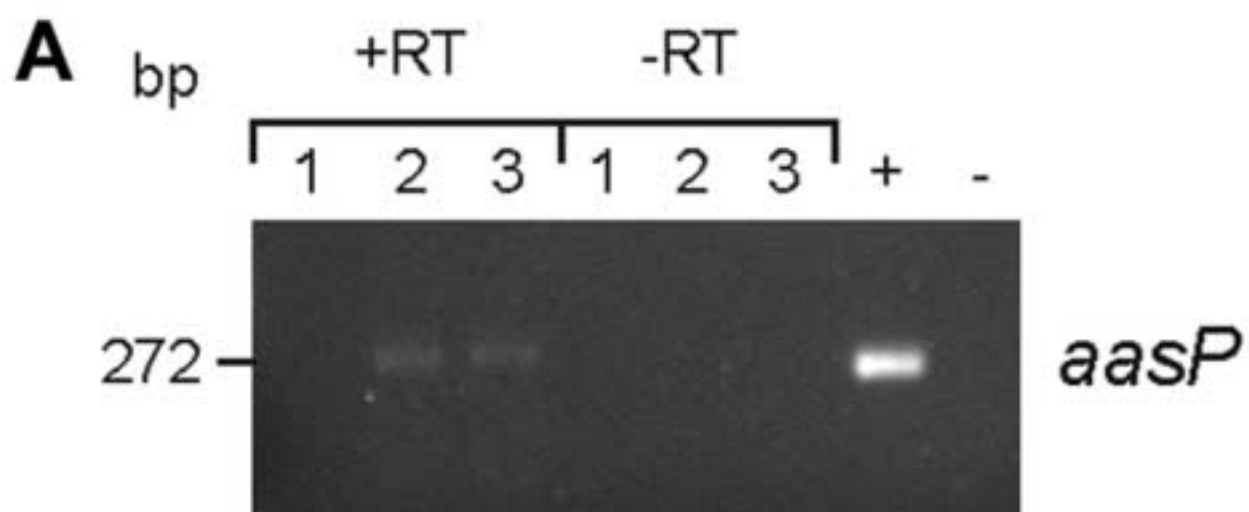
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- 17

TCCATAAAAAACTTGAGGTAAATCAAAAAAGATGAGAACTA
HlyX box
TTTGCAATAATAGACATCGTAAGTATAATCCCATTTTTTTA
TAAGGAGACTATAAATGCTTGAAAAATATTATTTTAATAAA
SD M L E K Y Y F N K
AasP ORF →

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AasP	GVSKNWCVAAPGNLAJ LNAI PDKL KPOYSLDKEEGTSLAIPVVTGALAI		363
Ssa1	GASKNWCVAAPGDLHVL GMADEHKKPOYGLTKEOGT SFSAPA TASLAV		363
SphB1	GOAQQWCLAAPSTAYLPGLDKDN---P-DSI HVEOGTSLSAPLVTGAAM		424
AusP	GI TAMWCLSAPYEASVRFRTN----P-----I Q AGTSFSAPI VTGTAAL		423
AasP	LKERFNLYLVPITQI RDTL LTTATDLGEGVDDKYGWGVI NI AKAI NGPSQF		413
Ssa1	LKERFDYLTATQI RDTL LTTATDLGEGVDN VY GWGLI NLK KAVNGP TQF		413
SphB1	VQDRFRWMDNDNLR TLLTTAODKGPYGVDPQY GWGVL DVGRAVDGPAQF		474
AusP	LLQKYPWMSNDNLR TLLTTAODI GAVGVDS KFGWGLLDAGKAMNGPAF		473



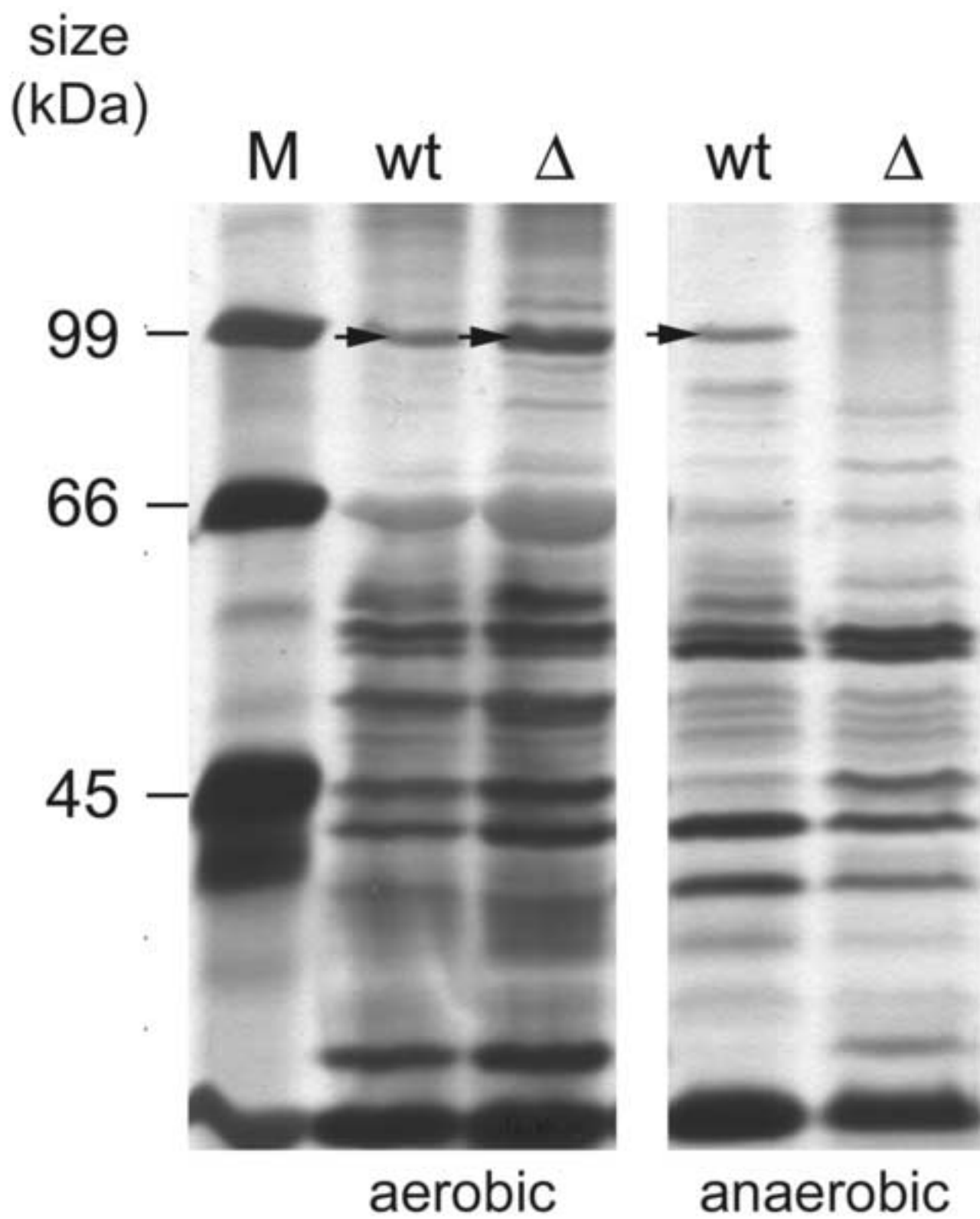


Table 1. Bacterial strains and primers

Strains and primers	characteristics	source (references)
Strains		
<i>A. pleuropneumoniae</i> AP76	<i>A. pleuropneumoniae</i> serotype 7 field strain	diagnostic unit of the department
<i>A. pleuropneumoniae</i> $\Delta hlyX$	<i>A. pleuropneumoniae</i> AP76 mutant lacking HlyX	(Baltes et al., 2005)
Primers		
oSCOTS-N9-1	5' GTGGTACCGCTCTCCGTCCGA-N9 3'	(Daigle et al., 2001)
oSCOTS-N9-2	5' CGGGATCCAGCTTCTGACGCA-N9 3'	(Daigle et al., 2001)
oSCOTS1	5' GTGGTACCGCTCTCCGTCCGA 3'	(Daigle et al., 2001)
oSCOTS2	5' CGGGATCCAGCTTCTGACGCA 3'	(Daigle et al., 2001)
oRRN16-1 oRRN16-2	5' TGGGTCGTAGGTTCAAATCC 3' 5' GCGTCAGTACATTCCCAAGG 3', Amplifies 5' end of 16s rRNA sequence, product size 990 bp	(Baltes and Gerlach, 2004)
oRRN16-3 oRRN16-4	5' TGTAGCGGTGAAATGCGTAG 3' 5' ACTTGAACCACCGACCTCAC 3' Amplifies 3' end of 16s rRNA sequence, product size 1000 bp	(Baltes and Gerlach, 2004)
oRRN16-6	5' CGTTGCATCGAATTAAACCA 3' upstream primer, amplifies a 262 bp fragment of 16s rRNA with primer oRRN16-3	this work
oRRN23-1 oRRN23-2	5' TTGGAAACAAGCTGAAACTGA 3' 5' GGACAGGAACCCTTGGTCTT 3'	(Baltes and Gerlach, 2004)

	Amplifies 5' end of 23s rRNA sequence product size 1480 bp
oRRN23-3	5' TCAGAAGTGCGAATGCTGAC 3'
oRRN23-4	5' CTGGCGAGACAACTGGTACA 3' Amplifies 3' end of 23s rRNA sequence, product (Baltes and Gerlach, 2004) size 1466 bp
oSER1	5' AAAACCCGTCGAACAGAATG 3'
oSER2	5' CATCTTTGGCAAGCTCATCA 3' Amplifies bp 294-565 of the <i>aasP</i> ORF, product this work size 272 bp

Table 1. Characteristics of bacterial strains and primers used in this study

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1 Table 2. Genes identified by SCOTS analysis

<i>ID^a</i>	<i>Protein^b</i>	<i>Id/Sim %^c</i>	<i>Span^d</i>	<i>accession no.</i>
	Surface			
2E12	Subtilisin-like serine protease AasP			ZP_00135639
	Regulatory			
1E9	Global anaerobic regulator HlyX			P23619
2B2	Transcription termination factor Rho			CAA76933
1A8	Transcription termination-antitermination factor NusA			ZP_00133986
	Transport			
1A1	SecA			ZP_00135175
1C4	Periplasmic xylose binding protein			ZP_00134919
1H3	Peptide transport periplasmic protein SapA			ZP_00348295
1E11	sodium/glutamate symporter			ZP_00135045
2C5	ABC-type transport system involved in resistance to organic solvents, ATPase component			ZP_00135102
2D8	ABC-type spermidine/putrescine transport system, permease component II			ZP_00133976
1A7	Predicted membrane protein			ZP_00134961
1F11	Mercuric transport protein			ZP_00134482
	Stress			
1B1	Superfamily II DNA and RNA helicases			ZP_00134573
1B8	EF-Tu*			ZP_00134976
1D4	DnaK			ZP_00134922
	Metabolism			
1A6	Streptomycin phosphotransferase (<i>Pseudomonas syringae</i> pv. <i>actinidiae</i>)	98/100	54	AAS21309
2A6	Beta lactamase			AAB24384
1G10	Dihydropteroate synthase			ZP_00134464
2C7	Tetracycline resistance protein TetB			ABA71359
2G11	Glyceraldehyde-3-phosphate dehydrogenase (<i>H. influenzae</i>)	95/98	74	P44304
2G4	Na ⁺ -transporting NADH:ubiquinone oxidoreductase, subunit NqrB*			ZP_00134697
1C10	Predicted permease			ZP_00135223
1A10	DNA primase			ZP_00134666
1B3	Acetyl-CoA carboxylase alpha subunit			ZP_00134950
1C12	F0F1-type ATP synthase, subunit b			ZP_00134544
1H7	30 S ribosomal protein			O31194
1D1	50 S ribosomal protein (<i>Haemophilus influenzae</i>)	92/98	44	P44344
1E7	TypA GTP binding protein (<i>Haemophilus influenzae</i>)	94/94	39	P44910
1D9	putative n-acetylmannosamine-6-phosphate 2-epimerase			ZP_00135289
1G3	RNA polymerase B*			ZP_00134648
1H4	tRNA pseudouridine synthase d			ZP_00204522
1G6	Actin-like ATPase involved in cell morphogenesis (MreB)			ZP_00134716
1G9	Triosephosphate isomerase (<i>Actinobacillus succinogenes</i>)	85/92	39	ZP_00732017
2C1	Methionyl-tRNA formyltransferase			ZP_00133763
2D1	Leucyl aminopeptidase			ZP_00134862
2E5	Glycosyl transferase (<i>Actinobacillus actinomycetemcomitans</i>)	46/67	71	BAA28141

1 asterisks denote genes also identified in a previous SCOTS analysis investigating *A.*
2 *pleuropneumoniae* gene expression in the porcine lung on day 7 post infection (Baltes and
3 Gerlach, 2004)
4 ^a clone number
5 ^b protein as identified in *A. pleuropneumoniae*, or homologues in other species, where
6 indicated.
7 ^c percentage of identity (Id) and similarity (Sim) on the amino acid level to proteins, given for
8 sequences for which homologues were found in species other than *A. pleuropneumoniae*
9 ^d number of amino acids from which the indicated percentage of identity and similarity was
10 derived.
11