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Identification of Genes Transcribed by *Actinobacillus pleuropneumoniae* in Necrotic Porcine Lung Tissue by Using Selective Capture of Transcribed Sequences

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Genes expressed by *Actinobacillus pleuropneumoniae* in necrotic porcine lung tissue were identified by selective capture of transcribed sequences analysis. In total, 46 genes were identified, 20 of which have been previously reported to be associated with in vivo expression or virulence in *A. pleuropneumoniae* or in other organisms.

The porcine respiratory tract pathogen *Actinobacillus pleuropneumoniae* presents a major problem to the swine industry due to its ability to persist in the host, surviving in tonsils as well as in sequestered necrotic lung tissue, which leads to the occurrence of subclinically infected carrier animals (6, 11, 18). Genes expressed by *A. pleuropneumoniae* only in the host are likely to play an important role in virulence and persistence. This is demonstrated by the finding that natural infection with *A. pleuropneumoniae* results in protection from reinfection with homologous and heterologous serotypes (7); the latter cannot be achieved by vaccination with bacterin vaccines commercially available (26). Therefore, the identification of genes expressed in these tissues would contribute significantly to the understanding of *A. pleuropneumoniae* disease.

To identify genes expressed by *A. pleuropneumoniae* in vivo, a variety of approaches have been successfully employed, like in vivo expression technology (IVET) (14, 36), signature-tagged mutagenesis (STM) (13, 34), and representational difference analysis (RDA) of *A. pleuropneumoniae* grown with the addition of bronchoalveolar lavage fluid (BALF) (4, 19). The latter technique is an ex vivo approach mimicking in vivo conditions. IVET is based on complementation of an attenuating auxotrophic mutation by gene fusion. In STM, a tagged transposon is used that disrupts genes by randomly inserting in the genome. Both STM and IVET analyses involve the infection of animals with a pool of mutants followed by recovery, selection, and comparative analysis of mutants. IVET then allows the identification of promoters switched on in vivo (14), while STM results in the identification of genes that are essential for survival in vivo (34).

The technique used for this work, selective capture of transcribed sequences (SCOTS), involves the capture of transcribed sequences by using biotinylated chromosomal DNA coupled to streptavidin-coated paramagnetic beads and a PCR-based subtractive hybridization with transcripts from culture-grown bacteria. It was originally described by Graham and Clark-Curtiss in 1999 for the identification of genes expressed by *Mycobacterium tuberculosis* upon growth in macrophages (16). The technique was subsequently used for the isolation of macrophage-activated *Mycobacterium avium* (23) and *Salmonella enterica* serovar Typhi genes (8). Recently it was demonstrated that the technique is sensitive enough to isolate bacterial genes expressed in tissues of infected animals (10).

While the enrichment principle of SCOTS is very similar to that of RDA, RDA alone cannot differentiate between host and bacterial cDNA. Additionally, RDA using BALF to induce differential gene expression will allow the identification of some genes expressed from *A. pleuropneumoniae* in epithelial lining fluid, but transcriptional differences as they occur in necrotic lung tissue will not be detected. The STM technique, an in vivo approach, identifies genes whose function cannot be complemented by either the single organism or the coinfecting mutants and are, therefore, essential for survival in vivo. Hence, its scope differs from SCOTS, which aims to identify genes that are upregulated in vivo but are not necessarily essential. The second in vivo approach, IVET, aims at the identification of promoters only switched on in vivo, thereby targeting some of the same genes found via SCOTS; however, it requires animal experiments under biosafety level 2 conditions, using pools of transformants. Further, in both in vivo techniques the different growth rates of individual transformants within the pool require a termination of the experiment within hours after infection. In IVET, clones carrying promoters that are switched on in later stages of infection may be eliminated before that time, and in STM the outgrowth of single clones restricts the diversity of the recovered pool, resulting in a large number of false positives, i.e., genes being falsely identified as essential in vivo. As neither of these techniques is applicable to the identification of genes involved in the later stages of the disease, the SCOTS analysis is a valid complementation and might facilitate further elucidation of *A. pleuropneumoniae* virulence, particularly in the postacute stages of infection.

For the study presented here, we employed SCOTS to identify genes expressed by *A. pleuropneumoniae* in necrotic por-

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

Strain, plasmid or primer	Characteristics and/or sequences	Source and/or reference
Strains		
A. pleuropneumoniae C1569	*A. pleuropneumoniae* serotype 9, clinical isolate	Diagnostic unit of the Department of Infectious Diseases
Escherichia coli TOP10 F′	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74 rec A1 deoR araD139* Δ(*araleu*)7697 *galU galK rpsL* (Str^r) *endA1 nupG*	TOPO TA Cloning (Invitrogen)
Plasmid		
pCR 2.1-TOPO	Topoisomerase I enhanced *E. coli* cloning vector	TOPO TA Cloning (Invitrogen) (35)
Primers		
M13 forward	5′ CAGGAAACAGCTATGAC 3′	Amersham Biosciences
M13 reverse	5′ GTAAAACGACGGCCAG 3′	Amersham Biosciences
oSCOTS-N9-1	5′ GTGGTACCGCTCTCCGTCCGA-N9 3′	8
oSCOTS-N9-2	5′ CGGGATCCAGCTTCTGACGCA-N9 3′	8
oSCOTS1	5′ GTGGTACCGCTCTCCGTCCGA 3′	8
oSCOTS2	5′ CGGGATCCAGCTTCTGACGCA 3′	8
oRRN16-1	5′ TGGGTCGTAGGTTCAAATCC 3′	This work
oRRN16-2	5′ GCGTCAGTACATTCCCAAGG 3′; amplifies 5′ end of 16s rRNA sequence; product size, 990 bp	
oRRN16-3	5′ TGTAGCGGTGAAATGCGTAG 3′	This work
oRRN16-4	5′ ACTTGAACCACCGACCTCAC 3′; amplifies 3′ end of 16s rRNA sequence; product size, 1,000 bp	
oRRN23-1	5′ TTGGAAACAAGCTGAAAACTGA 3′	This work
oRRN23-2	5′ GGACAGGAACCCTTGGTCTT 3′; amplifies 5′ end of 23s rRNA sequence; product size, 1,480 bp	
oRRN23-3	5′ TCAGAAGTGCGAATGCTGAC 3′	This work
oRRN23-4	5′ CTGGCGAGACAACTGGTACA 3′; amplifies 3′ end of 23S rRNA sequence; product size, 1,466 bp	
oADH7	5′ ACACGTAATGACGGCGGTA 3′	This work
oADH8	5′ CGAGTGGATTCACCCAATTT 3′; amplifies a 158-bp product from an *A. pleuropneumoniae* autotransporter adhesin; acc. no. ZP 00204542	
oFLPD1	5′ TTTCTCTGTAGAGATGGTTTGTGC 3′	This work
oFLPD2	5′ AACCCAATTAACCCAAATGGT 3′; amplifies a 154-bp product from an *A. pleuropneumoniae* fimbria-like protein; acc. no. NP 873737	

cine lung tissue at the end of the acute stage of infection (7 days postinfection). Pigs were infected with *A. pleuropneumoniae* C1569 (Table 1) by using an aerosol infection model described previously (1, 3, 4). The dose used was 10.8×10^4 CFU for four pigs, a dose which has been shown to reliably induce acute but not fatal disease. Samples of necrotic lung tissue from five pigs were obtained after 7 days of infection, and the samples were confirmed to be culture positive, with surface smears yielding dense to confluent growth on Columbia blood agar plates. Such samples typically contain 10^6 to 10^8 CFU per gram of tissue. Samples intended for RNA isolation were immediately preserved in RNAlater solution (Ambion Inc., Houston, Tex.) at the time of collection, RNA was prepared with RNEasy columns (QIAGEN, Hilden, Germany), and RNA integrity was verified by visualization on an agarose gel. For use in this experiment, RNA from five animals was then pooled. RNA samples intended for reverse transcription were treated with TurboDNAse (Promega, Mannheim, Germany) according to the manufacturer's instructions, and absence of genomic DNA was confirmed by PCR using primers oRN5-1 and oRN5-2, which amplify a 180-bp fragment of the dimethyl sulfoxide reductase gene (2). For subtraction, *A. pleuropneumoniae* C1569 RNA was prepared from liquid cultures that had been grown aerobically with shaking to an optical

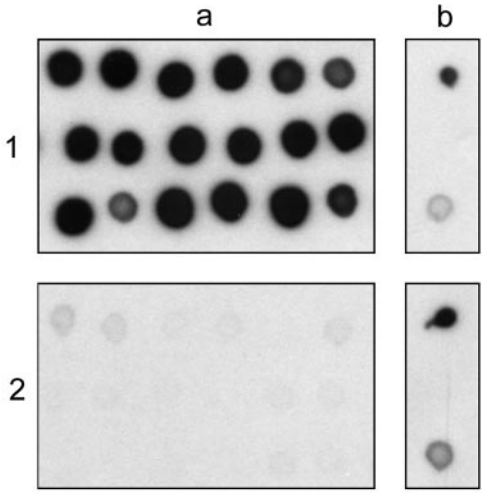

FIG. 1. Southern dot blot showing (a) SCOTS clones and (b) 1 μg (top) or 0.1 μg (bottom) of genomic *A. pleuropneumoniae* DNA as positive controls, hybridized to a radiolabeled probe generated from lung-derived (1) or culture-derived cDNA (2) after three rounds of normalization.

TABLE 2. Genes identified via SCOTS

Class	Clone(s)	Gene and/or possible function[a]	% Identity/ % similarity[b]	Span[c]	Reference(s)[d]	GenBank accession no.
Secreted	H7	ApxIVA var3 RTX toxin (*A. pleuropneumoniae*)			33	AAD01905
Cell surface	B5	Autotransporter adhesin, similar to Hsf in *H. influenzae* (*A. pleuropneumoniae*)			29, 39, 41	ZP_00204542
	E4	*flpD*, fimbria-like protein (*H. ducreyi*); *flp* genes are essential for microcolony formation in *H. ducreyi*	65/66	188	12, 31, 37	NP_873737
	B9	Outer membrane protein similar to immunogenic protein PomA of *P. multocida* (*A. pleuropneumoniae*)			13, 42	E47068
	F4	Outer membrane protein of unknown function (*A. pleuropneumoniae*)				ZP_00135264
Metabolism	A7	*guaA* GMP synthase (*A. pleuropneumoniae*), linked to virulence in various organisms, including *P. multocida*			12, 27, 32, 34	ZP_00134556
	A8	Diadenosine tetraphosphatase similar to ApaH in *S. enterica* serovar Typhimurium (*A. pleuropneumoniae*); linked to virulence in *H. parasuis* and serovar Typhimurium			21, 24	ZP_00134055
	A9	*fuc1*, L-fucose isomerase (*H. influenzae*); in *Bacteroides thetaiotaomicron*, the L-fucose metabolic pathway is coordinated with the production of fucosylated glycans in enterocytes	76/78	222	22, 25	P44779
	C2	*ptsB*, sucrose-specific phosphotransferase EII component (*P. multocida*); linked to virulence in serovar Typhimurium, *S. aureus*, and *H. influenzae*	83/85	216	28	NP_246785
	H3	Sugar phosphate permease (*A. pleuropneumoniae*)				ZP_00135166
	C7	Oxygen-sensitive ribonucleoside-triphosphate reductase (*A. pleuropneumoniae*)				ZP_00134686
	D2	Thiol:disulfide interchange protein, similar to DsbA in *H. influenzae* (*A. pleuropneumoniae*); essential for virulence factor expression in *P. aeruginosa*			17, 34	ZP_00134426
	D3	3-Polyprenyl-4-hydroxybenzoate decarboxylase (*A. pleuropneumoniae*)				ZP_00134932
	G6	Ribosomal protein RpS19 (*H. influenzae*)	100/100	90		P44385
	E12	Biotin-(acetyl-coenzyme A carboxylase) ligase (*A. pleuropneumoniae*)				ZP_00134488
	F12	Dethiobiotin synthetase (*A. pleuropneumoniae*)				ZP_00135280
	F11	NADH ubiquinone oxidoreductase subunit 5 (*A. pleuropneumoniae*)			34	ZP_00135039
	G8	SAM-dependent methyltransferase (*A. pleuropneumoniae*)			34	ZP_00134291
	A4	Uncharacterized conserved protein involved in oxidation of intracellular sulfur (*A. pleuropneumoniae*)				ZP_00133817
	A11	Transposase (*A. pleuropneumoniae*)				ZP_00134532
	D1	3-Oxoacyl-(acyl-carrier protein) reductase (*H. influenzae*)	78/82	238		P43713
	D4	Predicted GTPase (*A. pleuropneumoniae*)				ZP_00134954
	G11	ATPase (*A. pleuropneumoniae*)				ZP_00134371
	E9	Restriction-modification system (ALXA and HSDM, *M. haemolytica*); *M. haemolytica* AlxA activates leukotoxin transcription in *E. coli*	81/81	156	20	P95510
	F8	Molybdopterin biosynthesis protein (*H. ducreyi*)	84/86	174	34	AAP95073
	H6	Leucyl-tRNA synthetase (*A. pleuropneumoniae*)				ZP_00133841
	F9	Predicted hydrolase of alkaline phosphatase superfamily (*A. pleuropneumoniae*)				ZP_00134565
Regulatory	A3	*rpoB*, RNA polymerase subunit (*H. ducreyi*)	91/94	207		AAP96608
	B2	Cell shape-determining protein, similar to MreC (*A. pleuropneumoniae*)				ZP_00134717
	A10	Signal recognition particle protein (*H. influenzae*)	88/93	243		P44518
	H5	Molecular chaperone (*A. pleuropneumoniae*)				ZP_00134922
	H4	Putative transcriptional regulator (*A. pleuropneumoniae*)				ZP_00134590
Stress	C9	*tufA*, elongation factor (EF) Tu (*H. ducreyi*); EF-Tu acts as a fibronectin binding protein in *M. pneumoniae*, linked to virulence in *K. pneumoniae*	100/100	135	9, 40	NP_872680
Transport	A5	*apaA*, putative ABC transporter, immunogenic (*A. pleuropneumoniae*)			30	AF109148
	H1	*dmsA*, dimethyl sulfoxide reductase, catalytic subunit (*A. pleuropneumoniae*); deletion mutants are attenuated			2	AY138463

Continued on following page

TABLE 2—*Continued*

Class	Clone(s)	Gene and/or possible function[a]	% Identity/ % similarity[b]	Span[c]	Reference(s)[d]	GenBank accession no.
	C4	*hgbA*, hemoglobin-binding protein (*A. pleuropneumoniae*); enables iron uptake from hemoglobin			5, 38	AAO33396
	G1	Na+/H+ antiporter (*A. pleuropneumoniae*); essential for in vivo survival in *S. agalactiae*			27	ZP_00134319
	E1	Predicted metal-binding protein (*A. pleuropneumoniae*)				ZP_00135073
	E7	Membrane components of Kef-type potassium transport systems (*A. pleuropneumoniae*)				ZP_00134994
	F1	Anaerobic C4-dicarboxylate transporter (*A. pleuropneumoniae*)			34	ZP_00134386
	F2	*tbpA*, transferrin receptor; small subunit (*A. pleuropneumoniae*); upregulated and essential in vivo			1	CAA90896
	F5	ATPase; cation transport (*A. pleuropneumoniae*)				ZP_00135075
	G10	Di- and tricarboxylate transporters (*A. pleuropneumoniae*)				ZP_00135062
Unknown	B8	Hypothetical protein PA2218 (*P. aeruginosa*)	76/83	162		Q01609
	G7, F7[e]	*A. pleuropneumoniae;* no homology to known genes				

[a] Genes with highest similarity to SCOTS sequences, obtained from public databases, source organism in parentheses, genes names given where available. SAM, *S*-adenosylmethionine.
[b] Identity/similarity percentages apply to amino acids.
[c] Span means the number of nucleotides over which similarity was detected.
[d] Publications reporting the isolation of identical or similar sequences.
[e] Sequences have not been submitted as these are partial open reading frames only.

density at 600 nm of 0.4. RNA samples prepared from porcine lung tissue as well as RNA prepared from *A. pleuropneumoniae* C1569 grown under standard culturing conditions were subjected to reverse transcription with primer oSCOTS-N9-1 or oSCOTS-N9-2, consisting of a defined terminal sequence at the 5′ end and a random 9mer 3′ end (Table 1), according to the descriptions of Daigle et al. (8). Each cDNA population was subjected to three rounds of normalization, consisting of hybridization to biotinylated genomic *A. pleuropneumoniae* C1569 DNA that had been blocked beforehand using DNA representing 16S and 23S *A. pleuropneumoniae* rRNA sequences with four PCR products representing ribosomal 16S and 23S RNA sequences (Table 1). For enrichment of sequences preferentially transcribed or upregulated during growth in porcine lung tissue, biotinylated genomic *A. pleuropneumoniae* C1569 DNA was preblocked with PCR products representing rRNA sequences as well as cDNA obtained from culture-grown bacteria after three rounds of normalization. Three rounds of capture hybridization with normalized cDNA from *A. pleuropneumoniae* C1569 grown in the porcine lung were then performed. Lung-specific cDNAs were then cloned with the TOPO TA Cloning kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Cloned inserts were amplified by PCR, spotted on nylon membranes, and subjected to Southern dot blot analysis with [^{32}P]dCTP-labeled lung- and culture-specific cDNA pools obtained after three rounds of normalization, respectively. Dot blots were washed with 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate at 60°C. In order to allow comparison of the signal intensities in both blots, 1 and 0.1 μg of genomic *A. pleuropneumoniae* C1569 DNA spotted on nylon membranes were used as positive controls exposed alongside their corresponding SCOTS clone dot blots (Fig. 1). Signals were weaker or absent for all cloned inserts on the blot hybridized to the normalized culture-specific cDNA compared to signals on the blot hybridized to normalized lung-specific cDNA (Fig. 1), indicating that these sequences are either absent or present in much less abundance in cDNA prepared from *A. pleuropneumoniae* grown under standard culturing conditions.

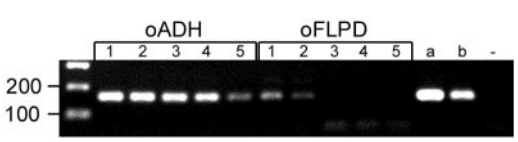

FIG. 2. PCR following reverse transcription of 5 μg of RNA derived from *A. pleuropneumoniae*-infected lung (lanes 1 to 5). Also shown is serial dilution of the cDNA template, ranging from undiluted (lane 1) to 1:10,000 (lane 5). Lanes a and b show positive controls using primer pairs oADH1/oADH2 (a) and oFLPD1/oFLPD2 (b), with chromosomal *A. pleuropneumoniae* DNA as template. −, No template.

Inserts were subjected to partial sequence analysis in a commercial laboratory (SeqLab, Goettingen, Germany). Sequence analyses were carried out using BLAST algorithms available in the HUSAR program package at the Deutsches Krebsforschungszentrum (dkfz) in Heidelberg, Germany. Sequence data were also compared to the database of the unfinished genomic sequence of *A. pleuropneumoniae* in a BLAST Search available at http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi. For reverse transcription-PCR, RNA was prepared and DNase treated as described above for SCOTS samples, and reverse transcription was carried out using 5 μg of RNA in the presence of 5 pmol of a gene-specific reverse primer.

We identified 46 genes differentially expressed by *A. pleuropneumoniae* in necrotic lung tissue (Table 2). Two of the sequences identified were confirmed to be part of *A. pleuropneumoniae* genes by database comparison but showed no homology to genes with known function. The remaining 44 genes identified by SCOTS analysis can be divided into six

functional groups: secreted proteins, cell surface, metabolism, regulatory, stress, and transport (Table 2).

Among the confirmed *A. pleuropneumoniae* sequences were four genes that are not expressed by *A. pleuropneumoniae* under standard culturing conditions but are present in vivo, such as the ApxIV toxin (33), the putative ABC transporter ApaA (30), the small subunit of the transferrin receptor, TbpB (15), and the catalytic subunit of dimethyl sulfoxide reductase (2) (Table 2). Further, some sequences identified in this study represent genes that have previously been identified in *A. pleuropneumoniae* via STM as being required for virulence (13, 34), such as GMP synthase, a thiol:disulfide interchange protein (DsbA), a molybdopterin cofactor synthesis protein, and an outer membrane protein similar to PomA of *Pasteurella multocida* (Table 2).

In addition, some of the identified genes have been reported to be expressed in vivo or to be involved in virulence in other organisms. Examples include a fimbria-like protein similar to FlpD of *Haemophilus ducreyi* which, in *H. ducreyi*, is necessary for microcolony formation and virulence in humans (31, 37); a diadenosine tetraphosphatase which plays a role in *Salmonella* spp. invasion (24); or hemoglobin binding protein HbgA, which is expressed in vivo in *P. multocida* (5) and under iron-deficient conditions in *A. pleuropneumoniae* (38). A homologue of the thiol:disulfide interchange protein identified in *A. pleuropneumoniae* via STM (34), DsbA, is required for the expression of the type III secretion system under low-calcium-inducing conditions, intracellular survival upon infection of HeLa cells, and twitching motility in *P. aeruginosa* (17).

Perhaps most importantly, a sequence similar to that of the high-molecular-weight autotransporter adhesin Hsf of *Haemophilus influenzae*, which is expressed by encapsulated *H. influenzae* strains and is homologous to the *H. influenzae* Hia adhesin (39), was identified. A Hia homologue is also expressed in vivo by *Neisseria meningitidis* (41). Autotransporter adhesins have not been characterized in *A. pleuropneumoniae* to date; however, a search of the unfinished genomic database for *A. pleuropneumoniae* revealed several putative autotransporter adhesin genes (GenBank accession numbers NZ_AACK01000066 and NZ_AACK01000041). In order to further confirm the results of the SCOTS analysis, transcription of the autotransporter adhesin and the *flpD* genes in porcine lung tissue was confirmed by reverse transcription-PCR using primers oADH1 and oADH2 as well as oFLPD1 and oFLPD2 (Fig. 2).

Our approach identified genes that may be upregulated in the host by a range of stimuli, including lack of iron, lack of oxygen, and host-specific factors. In order to confirm that we identified not only genes regulated by either iron deficiency or lack of oxygen, we repeated the enrichment procedure by using cDNA from bacteria grown under iron-deficient or anaerobic conditions, respectively, as previously described (2, 4). Thus, cDNA from bacteria grown under iron-deficient or anaerobic conditions after three rounds of normalization was used to preblock the biotinylated chromosomal *A. pleuropneumoniae* DNA before hybridization to lung-derived sequences. Subsequent analysis of differentially expressed sequences again identified the autotransporter adhesin and the ApxIV toxin, implying that their regulation is independent of iron deficiency and lack of oxygen.

For all other genes identified in this study and not mentioned explicitly above, putative functions as well as publications describing in vivo expression and/or involvement in virulence in *A. pleuropneumoniae* or other organisms are listed in Table 2. In total, 20 of the 46 identified genes have been linked to in vivo expression and/or virulence.

This finding strongly suggests that SCOTS analysis is a suitable tool for the study of gene expression in infected porcine lung tissue. Further experiments employing SCOTS analyses to identify transcriptional differences in bacteria at different localizations (i.e., tonsils and unaltered lung tissue) as well as in even later stages of infection might help to identify the molecular mechanisms for long-term colonization and give clues to its prevention.

Nucleotide sequence accession numbers. The sequences identified in the course of this work have been deposited in GenBank, and their numbers are listed in Table 2.

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