INFECTION AND IMMUNITY, Feb. 2004, p. 691–700 Vol. 72, No. 2
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.2.691–700.2004

Host Cell Contact-Induced Transcription of the Type IV Fimbria Gene Cluster of *Actinobacillus pleuropneumoniae*

Bouke K. H. L. Boekema,[1] Jos P. M. Van Putten,[2] Norbert Stockhofe-Zurwieden,[1] and Hilde E. Smith[1]*

Division of Infectious Diseases and Food Chain Quality, Institute for Animal Science and Health, ID-Lelystad, 8200 AB Lelystad,[1] and Department of Infectious Diseases and Immunology, Utrecht University, 3508 TD Utrecht,[2] The Netherlands

Received 13 May 2003/Returned for modification 29 July 2003/Accepted 5 November 2003

Type IV pili (Tfp) of gram-negative species share many characteristics, including a common architecture and conserved biogenesis pathway. Much less is known about the regulation of Tfp expression in response to changing environmental conditions. We investigated the diversity of Tfp regulatory systems by searching for the molecular basis of the reported variable expression of the Tfp gene cluster of the pathogen *Actinobacillus pleuropneumoniae*. Despite the presence of an intact Tfp gene cluster consisting of four genes, *apfABCD*, no Tfp were formed under standard growth conditions. Sequence analysis of the predicted major subunit protein ApfA showed an atypical alanine residue at position −1 from the prepilin peptidase cleavage site in 42 strains. This alanine deviates from the consensus glycine at this position in Tfp from other species. Yet, cloning of the *apfABCD* genes under a constitutive promoter in *A. pleuropneumoniae* resulted in pilin and Tfp assembly. Tfp promoter-*luxAB* reporter gene fusions demonstrated that the Tfp promoter was intact but tightly regulated. Promoter activity varied with bacterial growth phase and was detected only when bacteria were grown in chemically defined medium. Infection experiments with cultured epithelial cells demonstrated that Tfp promoter activity was upregulated upon adherence of the pathogen to primary cultures of lung epithelial cells. Nonadherent bacteria in the culture supernatant exhibited virtually no promoter activity. A similar upregulation of Tfp promoter activity was observed in vivo during experimental infection of pigs. The host cell contact-induced and in vivo-upregulated Tfp promoter activity in *A. pleuropneumoniae* adds a new dimension to the diversity of Tfp regulation.

Fimbriae or pili are filamentous polymeric structures that protrude from the bacterial cell surface (48). Type IV pili (Tfp) form a unique class of multifunctional fimbriae defined by shared structural features and a conserved biogenesis pathway. They are typically composed of thousands of core subunits with masses of 15 to 20 kDa that are polymerized into a fiber. During Tfp biogenesis, the major subunit is formed as a prepilin that is processed into mature pilin by a type IV prepilin peptidase. This enzyme removes the unique amino-terminal leader peptide and methylates the newly formed N-terminal amino acid residue prior to assembly of the subunits into pili. The genes and gene products required for Tfp biogenesis are remarkably conserved among the extremely diverse groups of gram-negative species that can produce Tfp (37). Tfp can display a diverse set of functions and may be involved in DNA uptake (16, 25, 47), adherence (10, 27, 28, 34, 37), protein export (9, 13, 16, 29, 30), twitching motility (23), and phage infection (44).

Besides the apparent conservation in biogenesis, architecture, and function, Tfp from different species can exhibit unique properties. The plasticity of Tfp ranges from variable length of the leader peptide to noted differences in the genetic regulation of Tfp expression among species (37, 46). The best-understood regulatory systems involve transcriptional modulation of the major subunit gene. In *Pseudomonas aeruginosa*, the PilS/R sensor-response regulator pair (12) and the alternative sigma factor σ^{54} (15) are essential for *pilA* transcription. In contrast, *Neisseria meningitidis pilE* utilizes a σ^{70} promoter (4) and is down-regulated upon cell contact by CrgA (5). Knowledge of the regulation of Tfp expression is of obvious importance in the dissection of the functions of Tfp in bacterial pathogenesis and their potential as a target for future infection intervention strategies.

In order to further explore the boundaries set to the plasticity of the Tfp system, we investigated the Tfp of *Actinobacillus pleuropneumoniae*. The Tfp of this respiratory pathogen may possess unique properties because of its high host specificity for pigs. *A. pleuropneumoniae* has been demonstrated to express fimbrial structures and to possess a 17-kDa protein that, based on its immunological cross-reactivity with Tfp of *M. bovis* and N-terminal amino acid sequence homology, was classified as belonging to the type IV family of pilus proteins. The potential to produce Tfp was further supported by the recent demonstration of a gene cluster that consists of four genes (*apfABCD*) that share homology at the deduced amino acid level with *pilABCD* of the Tfp gene family, although gene transcription was not demonstrated (33). Here we report the successful constitutive expression of fimbria subunits and of intact Tfp in *A. pleuropneumoniae* after placement of the cloned Tfp gene cluster behind a constitutive promoter. Additional experiments with promoter-reporter gene fusion constructs indicated that the Tfp cluster is preceded by an intact but tightly regulated promoter. Activation of native Tfp promoter activity required specific environmental conditions and

* Corresponding author. Mailing address: Division of Infectious Diseases and Food Chain Quality, Institute for Animal Science and Health, ID-Lelystad, P.O. Box 65, 8200 AB Lelystad, The Netherlands. Phone: 31-320-238023. Fax: 31-320-238961. E-mail: hilde.smith@wur.nl.

TABLE 1. *A. pleuropneumoniae* strains used in this study

Serotype[a]	Strain	Source
1	S4074	Reference
2	1536	Reference
3	1421	Reference
4	M62	Reference
5a	K17	Reference
5b	L20	Reference
6	Femø	Reference
7	WF83	Reference
8	405	Reference
9	CVI13261	Reference
10	D13039	Reference
11	56153	Reference
12	8329	Reference
1	N273	ID-Lelystad
2	N282	ID-Lelystad
1	HS25	Blackall[b]
1	HS57	Blackall
2	126023-1	ID-Lelystad
3	117559-5	ID-Lelystad
3	16169	ID-Lelystad
3	HS77	Blackall
3	126023-3	ID-Lelystad
5	J45	Inzana[c]
6	125739	ID-Lelystad
7	2827	ID-Lelystad
7	25535-2578	ID-Lelystad
7	HS30	Blackall
7	212:89-32159	Hilbink[d]
7	22:91-895	Hilbink
7	126398-165	ID-Lelystad
8	20044	ID-Lelystad
8	896	ID-Lelystad
9	HS17	Blackall
9	125943-191	ID-Lelystad
10	3177/89	Nielsen[e]
11	117559-1	ID-Lelystad
11	111290	ID-Lelystad
11	20492	ID-Lelystad
11	126219-2	ID-Lelystad
12	6807/90	Nielsen
2	118126G	ID-Lelystad
2	118126K	ID-Lelystad

[a] All listed strains are of biotype 1, except strains N273, N282, 118126G, and 118126K (biotype 2).
[b] P. Blackall, Animal Research Institute, Brisbane, Australia.
[c] J. Inzana, Virginia Polytechnic Institute and State University, Blacksburg.
[d] F. Hilbink, Central Animal Health Laboratory, Upper Hutt, New Zealand.
[e] R. Nielsen, State Veterinary Serum Laboratory, Copenhagen, Denmark.

was induced during the adherence of the pathogen to host epithelial cells and during experimental infection in pigs.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. Strains and plasmids used in this study are listed in Tables 1 and 2. *A. pleuropneumoniae* strains were grown on sheep blood agar plates containing 0.1% NAD (Calbiochem, La Jolla, Calif.) or in brain heart infusion medium (BHI) (Gibco BRL, Paisley, United Kingdom) containing 0.008% NAD (BHI-NAD) with or without 1.5% Bacto Agar (Becton Dickinson, Alphen aan den Rijn, The Netherlands). To study fimbria expression, *A. pleuropneumoniae* was grown on Luria-Bertani (LB) agar plates containing 0.008% NAD (LB-NAD) or in 5 ml of chemically defined medium (CDM) (11) in air, in CDM under microaerophilic conditions (composition: 6% O_2, 7% CO_2, 7% H_2, and 80% N_2, obtained with an Anoxomat WS8000 [Mart Microbiology, Lichtenvoorde, The Netherlands]), in tryptic soy broth (TSB) (Biotrading Benelux, Mijdrecht, The Netherlands) plus 0.008% NAD, or in LB medium plus 0.008% NAD. All *Escherichia coli* strains were routinely grown in LB with or without 1.5% Bacto Agar (Becton Dickinson). When appropriate, ampicillin (AMP) was added to the growth medium at a concentration of 100 μg/ml (*E. coli*) or 5 μg/ml (*A. pleuropneumoniae*). *E. coli* M15(pREP4) was grown in the presence of kanamycin at a concentration of 25 μg/ml. Bacteria were grown at 37°C unless indicated otherwise.

Preparation of inocula. For preparation of inocula, *A. pleuropneumoniae* strains were grown in 5 ml of BHI–0.008% NAD–AMP for 16 h. Bacteria were washed with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 2.8 mM K_2HPO_4, pH 7.2) and diluted to approximately 2×10^6 CFU/ml in PBS. The number of CFU before and after inoculation was determined by plating 10-fold dilutions in triplicate on BHI-NAD-AMP agar plates.

DNA transformation. For use in electro-transformation, *A. pleuropneumoniae* reference strain S4074 (serotype 1) was grown in 5 ml of TSB with 0.008% NAD (TSB-NAD) at 37°C with shaking at 120 rpm. After overnight growth, the culture was diluted 10-fold in TSB-NAD and incubated for 90 min at 37°C with shaking. Then, the bacteria were collected by centrifugation (5,500 × *g*, 10 min, 4°C), washed with 25 ml of chilled 274 mM sucrose–15% glycerol, and resuspended in 274 mM sucrose–15% glycerol to an optical density at 600 nm (OD_{600}) of 6.0. Fifty microliters of this cell suspension (which was kept on ice) was mixed with plasmid DNA (1 μg) and transferred to a prechilled electroporation cuvette (Bio-Rad, Richmond, Calif.) with an electrode distance of 2 mm. Electrical charges (2,500 V; capacitance, 25 μF; resistance of parallel resistor, 200 Ω) were delivered to ice-cold samples using a Gene-Pulser (Bio-Rad). Immediately after the electrical charge 900 μl of SOC medium (31) supplemented with 0.008% NAD was added, and the cells were allowed to recover at 37°C for 3 h with shaking. The cell suspension was plated onto BHI-NAD agar plates containing AMP (5 μg/ml) (BHI-NAD-AMP). Transformants were grown overnight in 5 ml of BHI-NAD-AMP and stored at −70°C in 50% glycerol in BHI. Transformation to *E. coli* was done according to the instructions supplied by the manufacturer.

PCRs. Oligonucleotides used for PCR and DNA sequencing were obtained from Isogen Biosciences (Maarsen, The Netherlands) or Gibco. Relevant oligonucleotides are listed in Table 3. Touch down PCR was carried out by using the AmpliTaq DNA polymerase kit reagents (Roche Molecular Systems, Inc., Branchburg, N.J.) according to the supplied protocol using primers 1024 and 1025. Each 50-μl PCR mixture contained 50 ng of template DNA, 15 pmol of (each) primer, 200 μM deoxynucleoside triphosphate mix, 1× PCR buffer, and 1.25 U of enzyme. Each sample was amplified using the following conditions: 10 min at 94°C; 10 cycles of 15 s at 94°C, 15 s at 55°C increased by 0.5°C per cycle, and 10 s at 72°C; 30 cycles of 15 s at 94°C, 15 s at 50°C, and 1 min at 72°C; and 7 min at 72°C.

Amplification of the complete fimbria operon was done by using the Expand High Fidelity kit (Roche) according to the supplied protocol using primers 25 and 26. Each 50-μl PCR mixture contained 50 ng of template DNA, 15 pmol of (each) primer, 200 μM deoxynucleoside triphosphate mix, 1× buffer, and 2.6 U of enzyme mix. Each sample was amplified using the following conditions: 2 min at 95°C; 10 cycles of 20 s at 94°C, 30 s at 55°C, and 270 s at 68°C; 20 cycles of 20 s at 94°C, 30 s at 55°C, and 270 s plus 5 s per cycle at 68°C; and 10 min at 72°C.

Standard PCR was carried out by using the Takara ExTaq kit reagents (Takara Shuzo Co., Ltd., Otsu, Shiga, Japan) according to the supplied protocol. Each 50-μl PCR mixture contained 50 ng of template DNA, 15 pmol of primer, 200 μM deoxynucleoside triphosphate mix, 1× PCR buffer, and 1.25 U of enzyme. Each sample was amplified using the following conditions: 10 min at 94°C; 30 cycles of 15 s at 94°C, 30 s at 60°C, and 30 s at 72°C; and 7 min at 72°C. All PCRs were performed on a Primus 96 apparatus (MWG Biotech AG, Ebersberg, Germany).

DNA manipulations, Southern blotting, and hybridization. Plasmid DNA was isolated by using the Miniprep or Midiprep Wizard kit (Promega Corporation, Madison, Wis.). Genomic DNA was isolated as described by Sambrook et al. (31). DNA ligations were done by using the rapid ligation kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim, Germany). For use in Southern or spot blot hybridization, PCR products were labeled with [α-^{32}P]CTP via random-primed labeling (Boehringer Mannheim). For spot blotting, 3 μl of plasmid DNA or 3 μl of culture was spotted on Genescreen Plus (NEN Life Science Products, Boston, Mass.), denatured with 0.4 M NaOH–1 M NaCl (two times 5 min), and neutralized in 2× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate). For Southern blotting, approximately 20 μg of bacterial genomic DNA was digested with *Eco*RI, subjected to electrophoresis in a 0.8% agarose gel, and transferred to Genescreen Plus by standard procedures (31). Radioactive labeled amplicons were boiled for 10 min, chilled in ice, and used as probes. Blots were incubated with the labeled probes for 16 h at 65°C in hybridization solution (342 mM Na_2HPO_4, 158 mM NaH_2PO_4, 1 mM EDTA, 7% [wt/vol] sodium dodecyl sulfate [SDS]). The membranes were washed twice (30 min, 65°C) with washing solution (27 mM Na_2HPO_4, 13 mM NaH_2PO_4, 1 mM EDTA)

TABLE 2. *E. coli* strains and plasmids used in this study

E. coli strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
DH5αF′	Library of partially *Sau*3AI-digested DNA fragments of *A. pleuropneumoniae* strain AP76 of serotype 7 DNA in plasmids pGH432 and pGH433	Gerald F. Gerlach, Tierärtzliche Hochschule, Hannover, Germany (2)
XL2-Blue	Used for plasmid construction and analysis	Stratagene, La Jolla, Calif.
M15(pREP4)	Used for analysis of expression vectors	Westburg, Leusden, The Netherlands
Plasmids		
pQE30	Expression vector	Westburg
pQE-ApfA	0.5-kb *Bam*HI-*Sph*I fragment containing *A. pleuropneumoniae apfA* lacking part of signal peptide in frame with N-terminal HIS tag in pQE30	This study
pGEM7	Used for cloning	Promega
pUC18	Used for cloning	Gibco
pKUN	Used for cloning	18
pGH432	Vector used for construction of a library of partially *Sau*3AI-digested DNA fragments of *A. pleuropneumoniae* strain AP76 of serotype 7	Gerald F. Gerlach (2)
pGH433	Vector used for construction of a library of partially *Sau*3AI-digested DNA fragments of *A. pleuropneumoniae* strain AP76 of serotype 7	Gerald F. Gerlach (2)
pTF86	Promoter-trap vector that contains, in sequence, the T4 terminator, a unique *Bam*HI site, and a promoterless copy of the *Vibrio harveyi luxAB* genes in pGZRS19	Martha Mulks, Michigan State University, East Lansing (6)
pSD2	Active SD2 promoter of *A. pleuropneumoniae* serotype 1 in promoter-trap vector pTF86	Martha Mulks (6)
pTF-F	Promoter region in *apfA* orientation of *A. pleuropneumoniae* serotype 1 in promoter-trap vector pTF86	This study
pTF-R	Promoter region in *radA* orientation of *A. pleuropneumoniae* serotype 1 in promoter-trap vector pTF86	This study
pUC-ApfABCD	3.9-kb *Xba*I-*Bam*HI fragment containing *A. pleuropneumoniae apfABCD* operon, including RBS but lacking promoter in pUC18	This study
pUC-SD2-ApfABCD	SD2 promoter upstream of fimbria operon in pUC-ApfABCD	This study
pGZRS19	*E. coli-A. pleuropneumoniae* shuttle vector	Susan West, University of Wisconsin—Madison (45)
pGZRS-F1	Fimbria operon downstream of SD2 promoter in pGZRS19	This study

containing 5% SDS and twice (30 min, 65°C) with the same solution containing 1% SDS.

Cloning. In order to verify the specificity of the ApfA peptide antiserum, the *apfA* gene was PCR amplified with primers 9 and 10 (Table 3; Fig. 1) and cloned in frame with a His tag at the amino terminus (Fig. 2A) in the expression plasmid pQE30, generating pQE-ApfA. pQE-ApfA was used to transform *E. coli* M15(pREP4). Expression was induced by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside).

The entire fimbria operon of *A. pleuropneumoniae* serotype 1 containing the putative ribosome binding site but lacking its own putative promoter sequence was amplified with primers 25 and 26 (Table 3; Fig. 1) and the High Fidelity kit. The resulting PCR product was cloned in pUC18 with *Xba*I and *Bam*HI gener-

TABLE 3. Oligonucleotides used in this study

Oligonucleotide (restriction site)	Location (nt)[a]	Use	Sequence (5′ to 3′)[b]
1024	1167–1198	Touch down PCR on fimbrial subunit	AAAAAAGGGTTTACATTAATCG
1025	1379–1354	Touch down PCR on fimbrial subunit	GCTIIAATICCITTTTGTCCICCIITAC
FwG	1742–1768[c]	Insert PCR on pGH432 and pGH433	CGGCCAAGCTTACTCCCCATCCCC
RevG	1947–1921[c]	Insert PCR on pGH432 and pGH433	CCACTCCCTGCCTCTGTCATCACG
8	135–156	Sequence analysis of cleavage site	TGTTCGGTCATGGCAAATACGC
9 (*Bam*HI)	1155–1175	Cloning of *apfA*	CGGGATCCCGTATTCGACCGCTTACTAACGCG
10 (*Sph*I)	1642–1664	Cloning of *apfA*	ACATGCATGCATGTGCCACTGTTCCTCGGAAATCCGG
25 (*Xba*I)	1037–1059	Cloning of *apfABCD*	GCTCTAGAGCGATACGGATCGCAGAAGTCGG
26 (*Bam*HI)	4882–4902	Cloning of *apfABCD*	CGGGATCCCGCCGATTCCACCGGTTAAACCG
29 (*Bam*HI)	1177–1158	Cloning of promoter region	CGGGATCCCGAAACGCGTTAGTAAGCGGTCG
30 (*Bam*HI)	791–813	Cloning of promoter region	CGGGATCCCGCATATCCGCTGAAGCGGTCGC

[a] According to the operon sequence of *A. pleuropneumoniae* strain AP76 determined for this work (accession number AY235719).
[b] Inosine (I) was incorporated to reduce specificity. Underlined nucleotides are not exact matches to the sequence and were altered to add restriction enzyme sites.
[c] Location in plasmids pGH432 and pGH433, used for sequence analysis of inserts of *A. pleuropneumoniae* genomic DNA.

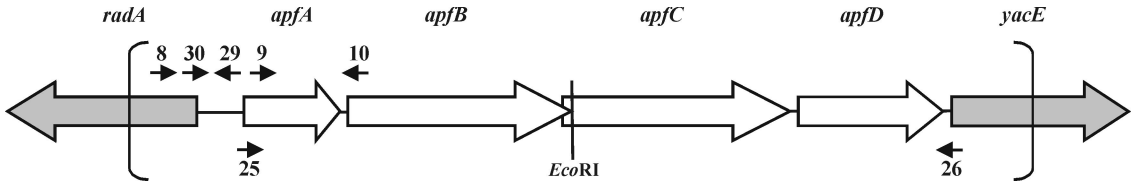

FIG. 1. Arrangement of the type IV fimbria operon in *A. pleuropneumoniae*. The region between accolades was completely sequenced. Open arrows represent type IV fimbria genes, while filled arrows represent genes that are not involved in fimbria biogenesis. Small black arrows with numbers indicate the positions of primers.

ating pUC-ApfABCD. An *Eco*RI fragment from pSD2 containing the transcription terminator T4 and constitutive *A. pleuropneumoniae* promoter SD2 was subcloned in pGEM7, and a 600-bp fragment containing T4/SD2 was cloned with *Hin*dIII and *Xba*I upstream of the fimbria operon in pUC-ApfABCD, generating pUC-SD2-ApfABCD. The fragment containing T4/SD2 and the fimbria operon was subsequently cloned in pGZRS19 with *Hin*dIII and *Bam*HI, generating pGZRS-F1. pGZRS19 and pGZRS-F1 were used to transform *E. coli* XL2-blue as well as *A. pleuropneumoniae* S4074.

A PCR product with primers 29 and 30 (Table 3; Fig. 1) containing the fimbria promoter region of *A. pleuropneumoniae* S4074 was cloned in pKUN with *Bam*HI, generating pKUN-F. A 320-bp *Bam*HI fragment from pKUN-F was cloned in front of the promoterless *luxAB* genes in pTF86 generating pTF-F (orientation for the fimbria promoter) and pTF-R (orientation for the *radA* promoter). The orientations of the inserts in pTF86 were confirmed by restriction analysis with *Eco*RI and *Bgl*II.

Sequence analysis was performed on inserts in plasmids pQE-ApfA, pGZRS-F1, pTF-F, and pTF-R.

DNA sequencing and analysis. DNA sequences were determined by using the Dye Terminator cycle sequencing ready reaction kit (PE Biosystems, Warrington, United Kingdom) in an ABI 373A DNA sequencer (Applied Biosystems, Foster City, Calif.). Reaction mixtures contained 500 ng of template plasmid DNA or 20 ng of PCR product, 8 μl of reaction mixture, and 3.2 pmol of primer in a 20-μl volume. Alternatively, DNA sequences were determined by Plant Research International (Wageningen, The Netherlands) by using the Big-Dye Terminator mix (version 2.0; Applied Biosystems). Reactions contained 500 ng of template plasmid DNA, 4 μl of reaction mix, and 10 pmol of primer in a 10-μl volume. Cycle sequencing reactions were performed on a Primus 96 apparatus (MWG Biotech). In all cases, both strands were sequenced. Primers FwG and RevG (Table 3) were used for sequence analysis of inserts in plasmids pGH432 and pGH433. Sequence analysis was performed using the DNAstar software package (DNAstar Inc., Madison, Wis.). To search for homologies, the nucleotide and amino acid sequences were compared with sequences in the GenBank databases by using BLAST (1).

SDS-polyacrylamide gel electrophoresis and Western blot analysis. Production of fimbria subunits was analyzed by SDS-polyacrylamide gel electrophoresis (17.5% polyacrylamide) and Western blotting. Blots were immunostained with six-His-tagged monoclonal antibody (anti-His; Clontech Laboratories, Palo Alto, Calif.) or polyclonal antifimbria peptide serum (Eurogentec, Seraing, Belgium). The antifimbria peptide serum was raised in mice against a short synthetic peptide with amino acid sequence CSGGQNGVRKMTELR from ApfA (Eurogentec).

Lux analysis. Quantitative analysis of Lux expression was performed on a Victor 1420 multilabel counter (Wallac, Turku, Finland). *N*-decyl aldehyde (Sigma Chemical Co., St. Louis, Mo.) substrate was made by dissolving a 20-mg/ml concentration of Essentially Fatty Acid Free bovine serum albumin (Sigma) in 1 ml of H_2O with *N*-decyl aldehyde (1 μl/ml). This mixture was incubated in a glass screw-cap test tube for 30 min in a sonicating water bath at room temperature to disperse the *N*-decyl aldehyde into micelles. For Lux analysis, 20 μl of bacterial lysate was mixed with 20 μl of substrate in white Polysorb luminescence plates (Nunc GmbH & Co. KG, Wiesbaden, Germany). This mixture was then read with normal emission aperture, a delay of 5 s, and a counting time of 10 s. Luminometer readings (counts per second [CPS]) were normalized to the number of bacteria in the sample as determined by plate counts on selective media (μCPS per CFU) or to the OD_{600} for pure cultures of bacteria. An OD_{600} of 1.0 equals approximately 10^9 CFU/ml.

Promoter activity in vitro. To investigate promoter activity in vitro, overnight cultures of *A. pleuropneumoniae* strains grown in BHI–0.008% NAD–AMP were diluted 10 times in 5 ml of BHI–0.008% NAD–AMP and incubated for 3 h at 37°C without shaking. Bacteria were washed once with test medium, resuspended, and incubated in test medium for 2 h at 37°C without shaking. Test media included BHI–0.008% NAD, CDM, CDM–20 μM $FeSO_4$, and CDM–0.03% NAD, and all media were supplemented with AMP at a concentration of 5 μg/ml. OD_{600} was determined and 2.5 ml of culture was centrifuged for 10 min at 5,500 × *g* at 4°C, and pellets were resuspended in 40 μl of lysis buffer (50 mM KCl, 2.5 mM $MgCl_2$, 1.8 μM SDS, 15 mM Tris-HCl) and directly used for Lux quantitation.

Promoter activity in the presence of LEC. The isolation and culture of porcine lung epithelial cells (LEC) is described elsewhere (3). Overnight bacterial cultures were centrifuged and the pellets were resuspended in Dulbecco's modified Eagle's medium (Gibco). Cell monolayers of LEC were infected at a multiplicity of infection of 1,000 (with approximately 10^8 CFU/ml) in the presence of AMP (5 μg/ml) and were incubated at 37°C in a 5% CO_2 atmosphere. After 2 h, supernatant medium with nonadherent bacteria was removed and kept at 4°C. LEC were washed four times with 3 ml of PBS. Adherent bacteria were released by treating the cell monolayers with 1% Triton X-100 in PBS (for 1 min). Controls consisted of bacteria incubated with medium alone. For additional controls, bacteria that were incubated with medium alone were centrifuged for 10 min at 5,500 × *g* at 4°C and were resuspended in 1% Triton X-100 in PBS (for 1 min). The numbers of CFU in supernatant medium and medium alone and of Triton X-100-treated bacteria and adherent bacteria were determined by plating 10-fold dilutions in triplicate on BHI-NAD-AMP agar plates. One milliliter of each suspension was centrifuged for 10 min at 5,500 × *g* at 4°C. The pellets were resuspended in 20 μl of lysis buffer and directly used for Lux quantitation.

Promoter activity in vivo. Animal experiments were performed in three similar, consecutive trials in pigs in good health free of *A. pleuropneumoniae*. The pigs were about 5 weeks of age and were housed in sterile stainless steel isolators. For endobronchial infection, pigs were anesthetized with a combination of azaperone (Stresnil; Jansen Pharmaceutica B.V., Tilburg, The Netherlands) and ketamine hydrochloride (Ketamine; Kombivet B.V., Etten-Leur, The Netherlands). Inoculation was performed as previously described (42). Briefly, a catheter with an outer diameter of 2.2 mm was advanced through the trachea deep into the bronchi and 5 ml of bacterial suspension was slowly administered. Three pigs per group were inoculated with approximately 10^7 CFU of *A. pleuropneumoniae* S4074 containing plasmids pTF86, pTF-F, or pSD2. The average inoculum contained 8.54×10^6 CFU. Two hours postinfection, pigs were anesthetized by intravenous injection of pentobarbital and exsanguinated. The lungs were excised, and three tissue specimens of approximately 1 cm^3 were taken from both distal caudal lung lobes for Lux analysis. Tissues were minced with scalpels, and 1.5 ml of PBS was added. Tissue suspensions were transferred to 5-ml tubes, mixed for 5 s, and centrifuged for 5 min at 200 × *g* to remove large clumps of tissue. Bacterial concentrations of the supernatant were determined by plating 10-fold dilutions on BHI-NAD-AMP agar plates. One milliliter of supernatant was centrifuged for 5 min at 10,000 × *g*. The pellets were resuspended in 100 μl of lysis buffer and directly used for Lux quantitation. For Lux analysis, the bacterial lysate was mixed with 100 μl of *N*-decyl aldehyde. All animal experiments were approved by the ethical committee of ID-Lelystad.

Electron microscopy. Cultures were examined for the presence of fimbriae by negative staining. Bacteria were absorbed on carbon-coated collodion nickel grids from agar plates or suspensions. The grids were then floated three times for 5 s on a solution of 1% methylamine tungstate (Bio-Rad). After the staining procedure, the specimens were viewed in a Philips CM10 electron microscope.

Statistics. Student's *t* test was used for statistical analyses. *P* values of ≤0.05 were considered significant.

Nucleotide sequence accession numbers. The nucleotide sequences of the Tfp gene clusters of strains S4074 and AP76 are available at GenBank under accession numbers AY235718 and AY235719.

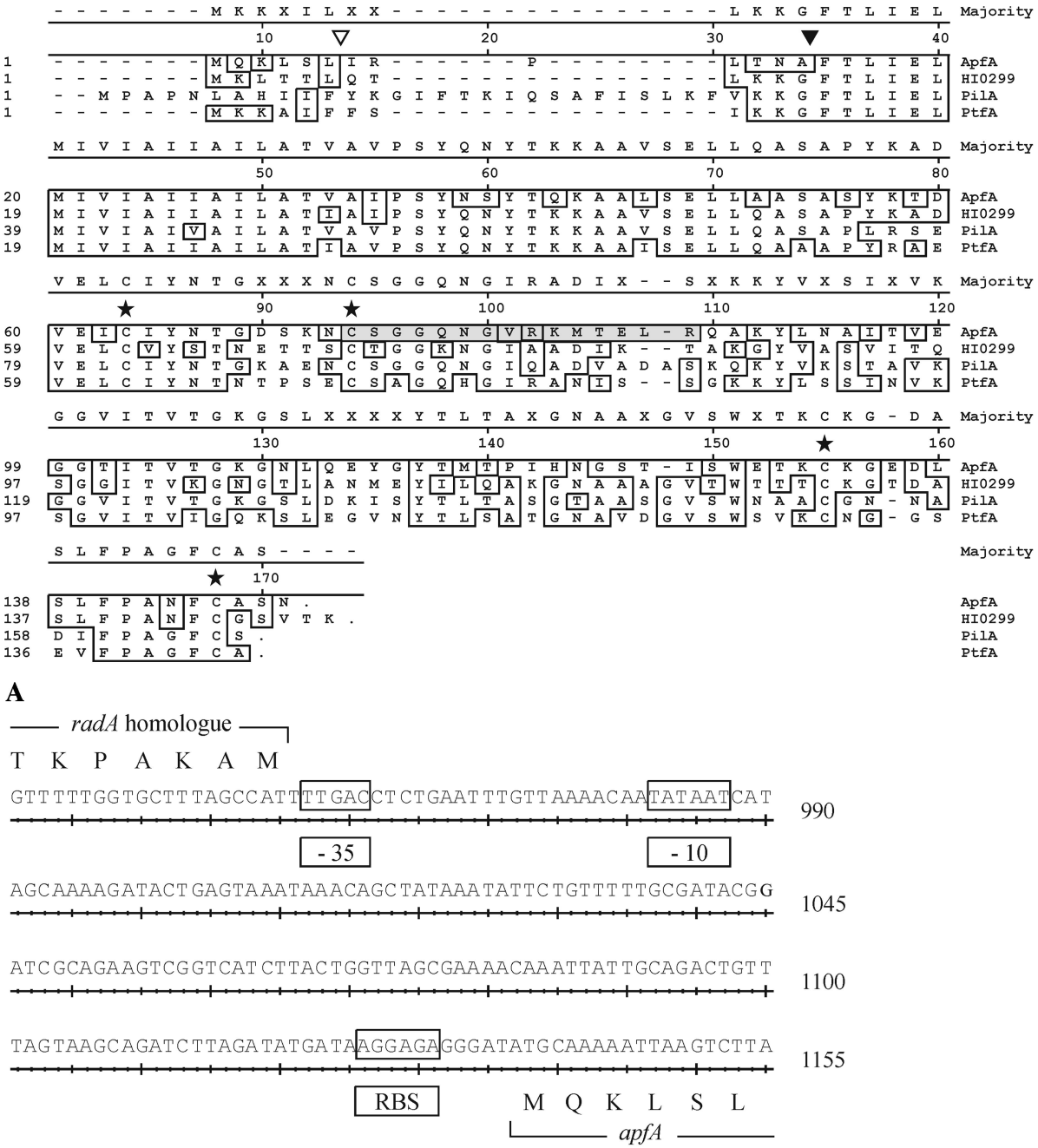

FIG. 2. (A) Alignment of amino acid sequences of type IV fimbria subunits of *A. pleuropneumoniae* strain S4074 of serotype 1 and of three other *Pasteurellaceae*. Residues that are identical to the consensus are boxed. The putative cleavage site is indicated by a filled triangle. The position of the fusion of ApfA to the His tag in pQE-ApfA is indicated by an open triangle. Conserved cysteine residues are indicated by stars. A synthetic peptide with the amino acid sequence of residues 73 to 87 (CSGGQNGVRKMTELR [shaded]) of ApfA was used to immunize mice. GenBank accession numbers are as follows: for *A. pleuropneumoniae* ApfA, AY235718; for *H. influenzae* Rd HI0299, AAC21963.1; for *A. actinomycetemcomitans* PilA, AAF89188.1; for *P. multocida* PtfA, AAF61196.1. (B) Nucleotide sequence of the promoter region of the fimbria gene cluster from *A. pleuropneumoniae* strain S4074 of serotype 1. A putative σ^{70} promoter sequence (−35 and −10 box) and ribosome binding site (RBS) are boxed.

RESULTS

Cloning of the *A. pleuropneumoniae* fimbrial gene cluster. The *A. pleuropneumoniae* Tfp gene cluster was amplified in two steps. First, part of the major subunit gene was PCR amplified with primers based on a conserved fimbria subunit sequence of *Haemophilus influenzae* and *Actinobacillus actinomycetemcomitans* (primer 1025) and deduced from the previously determined N-terminal amino acid sequence of an *A. pleuropneumoniae* subunit (primer 1024). Inosines were incorporated

at seven positions in primer 1025 to reduce its specificity. Touch down PCR on genomic DNA from *A. pleuropneumoniae* reference strains S4074, 1536, and WF83 yielded bands of the expected size (220 bp) at annealing temperatures ranging from 35 to 40°C. DNA sequencing and subsequent analysis of the PCR fragments revealed 55% similarity at the amino acid level with the type IV fimbria subunits of *A. actinomycetemcomitans*, *H. influenzae* Rd, and *Pasteurella multocida*.

In order to obtain the entire *A. pleuropneumoniae* subunit gene (designated *apfA*) and possible flanking fimbrial genes, a DNA library of *A. pleuropneumoniae* serotype 7 was hybridized with the obtained *apfA* PCR fragments. Hybridizing clones were collected, and the entire DNA sequence of the inserts was determined. This procedure yielded a 5,303-bp DNA region that contained four complete and two partial open reading frames (ORFs) (Fig. 1). Similar data were obtained for reference strain S4074, serotype 1.

Properties of the major Tfp subunit gene, *apfA*. Sequence analysis indicated that the first complete ORF of the identified region was the *apfA* gene. The gene was 444 bp long and was predicted to encode a 15.9-kDa protein (Fig. 2A). The putative protein was 75 to 92% similar to the fimbria subunits of *H. influenzae*, *A. actinomycetemcomitans*, *P. multocida* (Fig. 2A), and *Haemophilus somnus* and identical to that of the putative ApfA protein of *A. pleuropneumoniae* serotype 2 (GenBank accession number AF302997). The deduced protein sequence of ApfA contains many of the features shared by type IV subunits in other gram-negative bacteria, except for the Ala residue at position −1 relative to the cleavage site (37) (Fig. 2A). Most known type IV prepilin-like leader sequences contain a glycine at this position (37) (Fig. 2A). PCR with primers 8 and 10 (Fig. 1; Table 3) and sequence analysis of 42 strains of *A. pleuropneumoniae* including HS25 (Table 1), a strain which has been reported to produce Tfp, confirmed that the Ala residue at position −1 was an intrinsic trait of the *A. pleuropneumoniae* subunit gene (data not shown). This analysis also revealed a stop codon at the predicted Gly residue 68 in *apfA* of the *A. pleuropneumoniae* reference strain WF83 of serotype 7.

Organization and characterization of the remaining of the Tfp gene cluster. Downstream of the *A. pleuropneumoniae* *apfA* gene three ORFs were identified which were designated *apfB*, *apfC*, and *apfD* (Fig. 1). These ORFs encode proteins with similarities to PilB, PilC, or PilD analogues of *A. actinomycetemcomitans*, *P. multocida*, *H. influenzae*, and *H. somnus* which are involved in fimbria assembly (17, 29, 40, 41). The predicted protein ApfD showed 45% similarity to PilD of *A. actinomycetemcomitans* but showed very low similarities to *P. multocida* and *H. influenzae* sequences. *apfD* putatively encodes a prepilin peptidase that cleaves the positively charged N-terminal signal peptide of ApfA and methylates the exposed phenylalanine residue (22, 38). ApfD contains two Asp residues (at positions 89 and 147) thought to be involved in the active site of prepilin peptidases (20) but lacks a cluster of four Cys residues found in other prepilin peptidases (35).

Analysis of the ORFs flanking *apfABCD* revealed a partial ORF at 181 bp upstream of *apfA* on the opposite strand that showed similarity to *radA*. This gene is involved in DNA repair and has no known relation with fimbria biogenesis (32). Downstream of *apfD*, a partial ORF was found that showed similarity to *yacE*. This gene encodes the enzyme dephosphocoenzyme A kinase, which catalyzes the final step in coenzyme A biosynthesis, the phosphorylation of the 3′-hydroxy group of the ribose sugar moiety (26). This gene also has no known relation with fimbria biogenesis.

Analysis of the intergenic sequences indicated that the *apfA* gene was preceded at 6 bp upstream of the putative start codon by the sequence AGGAGA (Fig. 2B), which resembles the AGGAGG consensus ribosomal binding sequence for *A. pleuropneumoniae* (6). A putative promoter with the sequence TT GAC (−35) and TATAAT (−10) with a spacing of 19 bp was identified at 180 bp from the ATG start codon (Fig. 2B). This promoter structure is similar to the consensus σ^{70} promoter structure TT(G/A)AA (−35) and TATAAT (−10) in *A. pleuropneumoniae* (6). None of the different fimbria genes was followed by a transcriptional terminator. This in conjunction with the spacing of the *apfABCD* genes suggests that the genes are arranged in an operon and may be cotranscribed.

***A. pleuropneumoniae* carries a single type IV fimbria operon.** To ascertain the presence of a single copy of *apfA* in the *A. pleuropneumoniae* genome, Southern blot hybridization was performed. Genomic DNA, isolated from *A. pleuropneumoniae* reference strains of serotypes 1 and 7 (S4074 and WF83) and field isolates HS25 and HS77, was digested with *Eco*RI, separated on agarose gel, and blotted. The blot was hybridized with a PCR product containing the first half of *apfA* as a probe. In all four strains, only one band hybridized with the probe (data not shown), indicating that only a single copy of *apfA* is present in the *A. pleuropneumoniae* genome of serotypes 1, 3, and 7. This was confirmed by homology searches using the complete ApfA or the signal peptide sequence of ApfA and the unfinished genome sequences of *A. pleuropneumoniae* serotypes 1, 5b, and 7 (available from GenBank).

Expression of the recombinant *A. pleuropneumoniae* Tfp. Electron microscopy on *A. pleuropneumoniae* strains S4074, WF83, HS77, and HS25 grown on LB-NAD agar plates yielded no fimbria-like structures protruding from the cell surface. Similar negative results were obtained for ApfA in Western blots when lysates of strains grown in a diverse set of media (including CDM) were probed with an antiserum raised against a synthetic ApfA peptide with the amino acid sequence CSGGQNGVRKMTELR (Fig. 2A). These data indicate that Tfp expression is either tightly regulated and/or that the identified operon is not functional.

To distinguish between these possibilities, a PCR product (obtained with primers 25 and 26 [Fig. 1]) containing the entire Tfp gene cluster of *A. pleuropneumoniae* S4074 but lacking its own promoter sequence was cloned in plasmid pGZRS19 downstream of the constitutive SD2 promoter. The resulting plasmid pGZRS-F1 was used to transform *E. coli* XL2-blue. Western blot analysis on whole-cell lysates of XL2-blue (pGZRS-F1) with the ApfA-specific antiserum demonstrated the presence of an approximately 15-kDa protein (Fig. 3, lane 5) that was absent from *E. coli* carrying the empty plasmid pGZRS19 (Fig. 3, lane 4). Western blot analysis with His-tagged ApfA and anti-His antibody (data not shown) confirmed that it was ApfA that was recognized by the peptide antiserum (Fig. 3, lanes 1 and 2). Similar results were obtained for *A. pleuropneumoniae* strain S4074 carrying plasmid pGZRS-F1 (Fig. 3, lane 8), indicating that Tfp subunits were

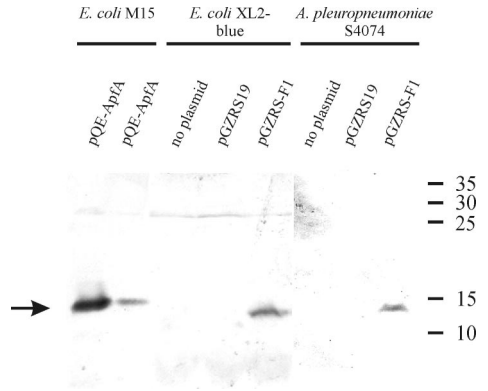

FIG. 3. Western blot analysis of fimbria subunit ApfA expressed in *E. coli* XL2-blue or *A. pleuropneumoniae* S4074 containing no plasmid, pGZRS19, or pGZRS-F1. Results for samples of *E. coli* M15(pQE-ApfA) are shown in lanes 1 and 2 (2 and 0.2 μl). The blot was stained with antifimbria peptide serum. The arrow indicates the position of the fimbria subunit ApfA (±15 kDa). Molecular size markers are indicated on the right (in kilodaltons).

produced. Electron microscopy demonstrated straight fimbriae protruding from the bacterial cell surface from the recombinant strain carrying the Tfp operon but not from the parent *A. pleuropneumoniae* S4074 (Fig. 4) or from *A. pleuropneumoniae* S4074(pGZRS19) (data not shown). Together, the data indicate that *A. pleuropneumoniae* carries an intact Tfp operon but that, at least under the laboratory growth conditions employed, the promoter activity may be insufficient to stimulate the formation of intact fimbriae.

Assessment of the Tfp promoter activity using *luxAB* gene reporter fusions. The molecular basis for the apparent absence of Tfp in *A. pleuropneumoniae* was further investigated with the use of promoter-reporter gene fusions. Sequence analysis of the putative Tfp promoter region suggested that a promoter is located between the *apfA* gene and the adjacent oppositely oriented *radA* gene (Fig. 2B). To determine possible promoter activity in this region, a PCR fragment containing this entire region of *A. pleuropneumoniae* S4074 was cloned in both orientations into reporter vector pTF86 in front of the promoterless *luxAB* genes, generating pTF-F (orientation for the fimbria promoter) and pTF-R (orientation for the *radA* promoter), respectively. As a positive control, plasmid pSD2, in which the *luxAB* genes are placed behind the constitutive *A. pleuropneumoniae* promoter SD2, was used (6). All plasmids (pTF86, pTF-F, pTF-R, and pSD2) were used to transform *A. pleuropneumoniae* S4074, and the level of expression of the *luxAB* genes was determined by measurement of Lux activity.

Growth of the various strains in different media (BHI-NAD-AMP or CDM-AMP) for 16 h or 10-fold dilutions of these cultures for an additional 1 to 4 h yielded no reproducible Lux activity for strain S4074 carrying the pTF-F plasmid or pTF86 (negative control). Under these conditions, strong positive signals were obtained for S4074 carrying pTF-R that carried the promoter region in the opposite (*radA*) orientation and S4074 carrying pSD2 (positive control) (data not shown). However, when bacteria at 3 h of exponential growth in BHI were collected by centrifugation, washed, and grown in CDM-AMP for an additional 2 h, S4074 carrying pTF-F did exhibit a luciferase activity of 4,678 μCPS/CFU, which was 26 times higher than that of the negative control strain S4074 carrying pTF86 ($P < 0.05$ [Table 4]). Similar experiments but with the strains grown in the final 2 h of incubation in BHI-NAD-AMP instead of in CDM indicated virtually no activity for the strain carrying the putative Tfp promoter (pTF-F), although good activity was observed for strains carrying pSD2 and pTF-R (Table 4). Extensive variation in the concentrations of potential regulatory compounds such as Fe^{2+} or NAD (between 0.0004 and 0.03%) in the media (43), or in growth temperature (33 versus 37°C), either had no effect or caused a minor increase (by a factor of 1.6 to 1.8) in Lux activity (data not shown). Together, these results strongly suggest that the *A. pleuropneumoniae* Tfp promoter is intact but active only under distinct and strictly defined environmental conditions.

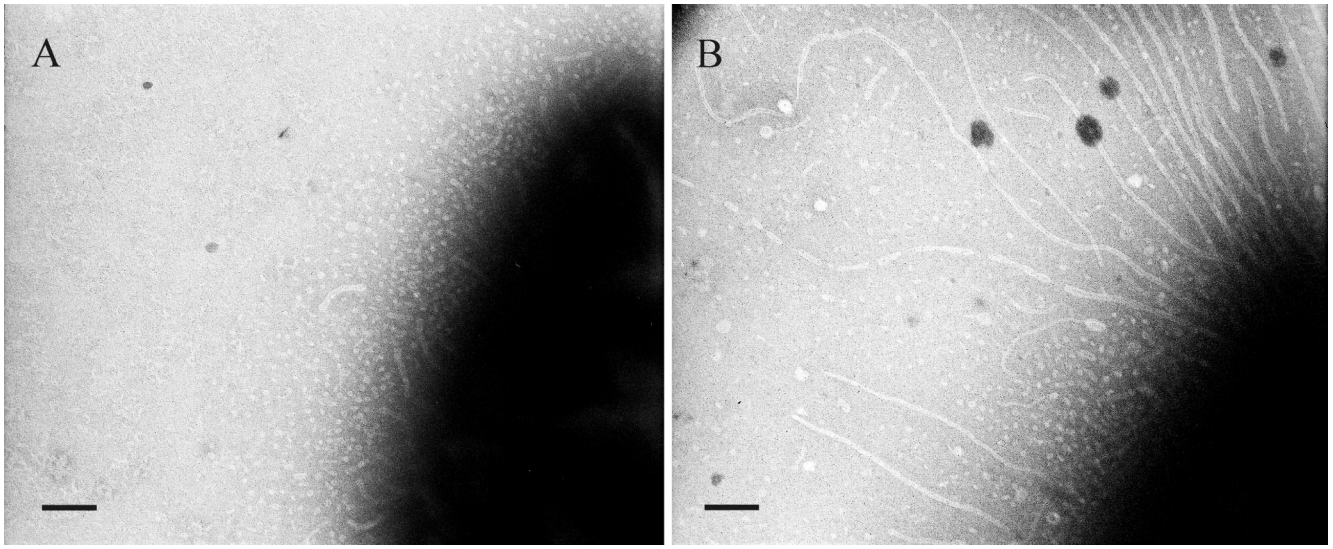

FIG. 4. Electron micrographs of *A. pleuropneumoniae* reference strain S4074 (A) and S4074(pGZRS-F1) (B). Bacteria were stained with methylamine tungstate and examined by electron microscopy as described in Materials and Methods. Bars represent 200 nm.

TABLE 4. In vitro promoter activity in *A. pleuropneumoniae* S4074

Plasmid	BHI-NAD-AMP		CDM-AMP	
	Mean Lux activity[a] ± SEM (μCPS/CFU)	Relative activity[b]	Mean Lux activity ± SEM (μCPS/CFU)	Relative activity
pTF86	90 ± 17	1	179 ± 15	1
pSD2	7,307 ± 6,651[c]	81.12	16,690 ± 986[c]	93.41
pTF-R	5,426 ± 659[c]	60.24	11,590 ± 723[c]	64.85
pTF-F	77 ± 14	0.85	4,678 ± 959[c]	26.18

[a] The results of three experiments in triplicate are shown.
[b] Relative Lux activity compared to pTF86.
[c] Significantly different from pTF86 in the same medium ($P < 0.05$).

Host cell contact-induced activation of the Tfp promoter. The apparent strict bacterial growth conditions required for activation of the Tfp promoter led us to assess its activity during infection of primary cultures of porcine LEC. The cells were inoculated with *A. pleuropneumoniae* strain S4074 carrying pTF86, pSD2, pTF-R, or pTF-F for a 2-h period. At this point, nonadherent bacteria, collected from the culture supernatant, and adherent bacteria, released from the host cells with 1% Triton X-100, were assayed for luciferase activity. Under these conditions, adherent S4074 carrying pTF-F demonstrated a luciferase activity of 1,523 μCPS/CFU (Table 5). This was substantially higher than the activity measured for the nonadherent bacteria isolated from the culture supernatant (221 μCPS/CFU, $P < 0.05$) and those from the adherent and nonadherent negative control S4074 carrying pTF86 with the promoterless *luxAB* genes (Table 5). Lux activities of the adherent and nonadherent positive controls S4074(pSD2) and S4074(pTF-R) were high under all conditions and ranged from 4,851 to 17,432 μCPS/CFU (Table 5). Treatment of bacteria with 1% Triton X-100 slightly reduced Lux activities of all four strains (data not shown). Together, these results clearly indicate that the Tfp promoter is active when bacteria adhere to the cell surface but not when they are present in the culture supernatant.

In vivo activity of the Tfp promoter. To validate our in vitro observations, the in vivo activity of the Tfp promoter was measured in an *A. pleuropneumoniae* pig infection model. *A. pleuropneumoniae* S4074 containing plasmids pTF86, pSD2, or pTF-F was used for endobronchial inoculation of pigs. Two hours after inoculation, pigs were sacrificed and the Lux activity of minced lung tissue was determined and related to the number of CFU (Table 6). As expected, the in vivo Lux activity of the strain with the promoterless *luxAB* genes, S4074(pTF86), was low (326 μCPS/CFU [Table 6]), and the in vivo Lux activity of the strain with the constitutive expressed *lux* genes in S4074(pSD2) was very high (22,601 μCPS/CFU [Table 6]). The in vivo Lux activity of S4074(pTF-F) carrying the Tfp promoter in the correct orientation was 1,176 μCPS/CFU. This was substantially higher than that of the negative control ($P < 0.05$) (Table 6) and of the activity determined after growth in BHI medium (Table 4). These data strongly suggest that the Tfp promoter is active in vivo during infection of lung tissue.

TABLE 6. In vivo promoter activity in *A. pleuropneumoniae* S4074

Plasmid	Lux activity[a] ± SEM (μCPS/CFU)	Relative activity[b]	In vivo/in vitro ratio (BHI)
pTF86	326 ± 60	1	3.62
pTF-F	1,176 ± 305[c]	3.61	15.27
pSD2	22,601 ± 2,814[c]	69.29	3.09

[a] Results of three tissue specimens of three pigs are shown.
[b] Relative Lux activity compared to pTF86.
[c] Significantly different from pTF86 ($P < 0.05$).

DISCUSSION

Tfp are important multifunctional bacterial surface organelles expressed by most gram-negative bacterial pathogens. Awareness is growing that regulation of Tfp expression is an essential quality enabling Tfp to function at the appropriate time. The environmental cues that control Tfp expression in the various species, however, are generally still poorly understood. In the present study, we investigated Tfp expression for the porcine respiratory pathogen *A. pleuropneumoniae*. This species carries a set of genes that shares features with the type IV pilin gene family, but this appears to result only rarely in the formation of Tfp (33, 43). We characterized the Tfp gene cluster and demonstrated that Tfp are formed when the cluster is placed behind a constitutive promoter. Promoter-reporter gene fusions showed that the *A. pleuropneumoniae* Tfp promoter is intact but tightly controlled by environmental conditions. Promoter activity was demonstrated to be induced upon contact of the bacteria with epithelial cells and in vivo during experimental infection of pigs.

The Tfp clusters of two different *A. pleuropneumoniae* strains consisted of four genes (*apfABCD*) separated by no or only small intergenic sequences and lacked apparent transcriptional terminator sequences. The overall organization of the gene cluster resembled that of the related bacterial pathogens *H. influenzae*, *A. actinomycetemcomitans*, and *P. multocida* (7,

TABLE 5. In vitro promoter activity in *A. pleuropneumoniae* S4074 in the presence of LEC

Plasmid	Bacteria treated with medium alone		Bacteria in supernatant of LEC		Bacteria binding to LEC	
	Mean Lux activity[a] ± SEM (μCPS/CFU)	Relative activity[b]	Mean Lux activity ± SEM (μCPS/CFU)	Relative activity	Mean Lux activity ± SEM (μCPS/CFU)	Relative activity
pTF86	318 ± 74	1	296 ± 73	1	93 ± 18	1
pSD2	15,890 ± 3,673[c]	50.06	15,940 ± 2,735[c]	53.95	17,430 ± 3,539[c]	188.33
pTF-R	9,230 ± 2,660[c]	29.07	4,851 ± 1,333[c]	16.42	5,970 ± 943[c]	64.49
pTF-F	214 ± 54	0.67	221 ± 58	0.75	1,523 ± 414[c]	16.45

[a] Results of six experiments are shown.
[b] Relative Lux activity compared to pTF86.
[c] Significantly different from pTF86 under the same conditions ($P < 0.05$).

8, 24) (unfinished genome of *A. actinomycetemcomitans* available from GenBank). It was remarkable that the *apfA* gene was preceded by *radA*, whereas in *A. actinomycetemcomitans*, *P. multocida*, and *H. influenzae* the major Tfp subunit gene is preceded by *ampD* (unfinished genome of *A. actinomycetemcomitans* available from GenBank) (7, 8, 24). The frequent clustering of the type IV subunit gene with *ampD* in other species and the fact that in other species *radA* is located elsewhere in the genome suggest that in *A. pleuropneumoniae* genomic rearrangements may have occurred that may have changed the characteristics of the Tfp promoter region and influenced the regulation of Tfp promoter activity.

Initially, the striking finding of an Ala residue at position −1 relative to the ApfA cleavage site, which was found to be a conserved feature among all 42 *A. pleuropneumoniae* isolates, was considered as a possible explanation for the apparent rare presence of Tfp at the surface of this pathogen. The consensus cleavage site of Tfp subunits consists of the residues Gly (−1), Phe (+1), and Glu (+5) (37). In *P. aeruginosa*, all but one mutation at residue −1 resulted in lack of processing of the major subunit PilA (36). Partial processing of PilA was observed with a mutation to Ala (−1), but this did not result in production of intact Tfp (36). A spontaneous mutant of *Neisseria gonorrhoeae* encoding a subunit containing Ser (−1) instead of Gly (−1) was also unable to assemble pili (19). Another type IV fimbrial subunit with an Ala (−1) is $PilE_L$ of *Legionella pneumophila*, which can be assembled in intact fimbriae (34). Thus, the consequence for Tfp expression of the presence of an Ala (−1) in the ApfA protein is difficult to predict. In our hands, cloning of the Tfp cluster into an expression vector in *A. pleuropneumoniae* resulted in the expression of ApfA and Tfp formation. This suggests that, at least with a strong promoter used, the Ala (−1) in ApfA does not preclude Tfp biogenesis. At this time we do not know whether the supposed prepilin peptidase ApfD of *A. pleuropneumoniae* has unique characteristics with respect to cleavage activity in comparison with other (PilD) prepilin peptidases or whether ApfA is cleaved at a reduced efficiency. It can be imagined that the latter may affect Tfp assembly when promoter activity is less strong. Putative prepilin peptidases of *Pasteurellaceae* appear to lack a cluster of Cys residues in the N-terminal half of the protein. Mutational analysis showed that the Cys residues are required for both cleavage and methylation activity of PilD (35). However, the role of these Cys residues in the activity of prepilin peptidases has been recently questioned. Some of the *pilD* mutants exhibited partial activity, and naturally occurring leader peptidases lacking the Cys residues can be fully functional (14, 35). Mutational analysis showed that two highly conserved Asp residues are absolutely required for protease activity, suggesting that type IV prepilin peptidases comprise a novel family of aspartic acid proteases (20).

Evidence that the Tfp promoter activity was subject to regulation was obtained when the putative Tfp promoter region of *A. pleuropneumoniae* S4074 was cloned into a promoter trap vector carrying the *luxAB* reporter genes. This strategy, which allowed direct monitoring of promoter activity, demonstrated that the DNA region preceding the Tfp operon carried two promoters: the Tfp promoter that turned out to have variable activity dependent on the environmental conditions and, on the opposite strand, the *radA* promoter that appeared to be constitutively active. The changes in luciferase activity observed with strains carrying this construct clearly indicated that promoter activity varied with the bacterial growth phase and the type of growth medium that was employed. Tfp promoter activity was found in cultures grown to mid- to late log phase in CDM but not when grown in BHI. These data likely provide the molecular basis for the reported variable presence of Tfp at the surface of *A. pleuropneumoniae* when these bacteria are grown in standard medium or in a CDM under microaerophilic conditions. It has been reported that in certain *A. pleuropneumoniae* serotypes (5a, 9, and 10) but not in others (serotype 2) NAD restriction is a critical factor for Tfp production (43). In our hands, variation in the concentration of NAD did not influence the activity of our (serotype 1) Tfp promoter. These data suggest the existence of serotype specific differences in the regulation of Tfp promoter activity. The exact signals that drive Tfp promoter activity in serotype 1 are unknown. We noticed that changes in temperature—which influence Tfp expression in, among others, *L. pneumophila* (21)—or the availability of iron had minor effects. These effects are probably not very specific and may well be related to concomitant changes in growth phase, which appear to influence Tfp promoter activity.

A key topic in the assessment of regulation of Tfp expression is the status of the system in the natural setting of an infection, i.e., during the adherence of the pathogenic bacteria to mucosal epithelial cells and during experimental infection in the legitimate host. *A. pleuropneumoniae* turned out to be an ideal model system to address this topic. The strong Tfp promoter activity measured for *A. pleuropneumoniae* bacteria that were adherent to primary cultures of LEC compared to that for nonadherent bacteria present in the culture supernatant strongly suggests that contact with epithelial cells is a trigger for Tfp production. Furthermore, our finding that the Tfp promoter was upregulated in vivo after endobronchial inoculation of pigs indicates that this regulation does occur in the natural host environment. The in vivo Lux activity appeared less than that observed for the bacteria adherent to the cultured lung cells, but this may be explained by the fact that we measured the total Lux activity in all bacteria (both adherent and nonadherent) present in the tissue samples. The finding that Tfp promoter activity is upregulated during contact with host cells may seem bizarre considering that Tfp often confer the initial bacterial attachment to host cells. For *N. meningitidis*, it has been demonstrated that the transcription of the Tfp-tip-associated adhesin PilC1 is upregulated in the presence of host cells (39). On the basis of the functions of type IV fimbriae in other bacterial pathogens, that the fimbriae of *A. pleuropneumonia*e play a role in the adherence and, possibly, at other stages of the infection must be considered a possibility. Whether Tfp of *A. pleuropneumonia*e are involved in other typical functions of Tfp like twitching motility, DNA uptake, protein secretion, or phage infection remains to be investigated. The nature of the environmental signals that drive the regulation of *apfABCD* transcription is still unknown. The Tfp-*luxAB* reporter system that we have developed may provide a good basis to take up this major challenge.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Baltes, N., W. Tonpitak, I. Hennig-Pauka, A. D. Gruber, and G. F. Gerlach.**

2003. *Actinobacillus pleuropneumoniae* serotype 7 siderophore receptor FhuA is not required for virulence. FEMS Microbiol. Lett. **220:**41–48.
3. **Boekema, B. K. H. L., N. Stockhofe-Zurwieden, H. E. Smith, E. M. Kamp, J. P. van Putten, and J. H. Verheijden.** 2003. Adherence of *Actinobacillus pleuropneumoniae* to primary cultures of porcine lung epithelial cells. Vet. Microbiol. **93:**133–144.
4. **Carrick, C. S., J. A. Fyfe, and J. K. Davies.** 1997. The normally silent sigma54 promoters upstream of the *pilE* genes of both *Neisseria gonorrhoeae* and *Neisseria meningitidis* are functional when transferred to *Pseudomonas aeruginosa.* Gene **198:**89–97.
5. **Deghmane, A. E., D. Giorgini, M. Larribe, J. M. Alonso, and M. K. Taha.** 2002. Down-regulation of pili and capsule of *Neisseria meningitidis* upon contact with epithelial cells is mediated by CrgA regulatory protein. Mol. Microbiol. **43:**1555–1564.
6. **Doree, S. M., and M. H. Mulks.** 2001. Identification of an *Actinobacillus pleuropneumoniae* consensus promoter structure. J. Bacteriol. **183:**1983–1989.
7. **Doughty, S. W., C. G. Ruffolo, and B. Adler.** 2000. The type 4 fimbrial subunit gene of *Pasteurella multocida.* Vet. Microbiol. **72:**79–90.
8. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, et al.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269**:496–512.
9. **Fullner, K. J., and J. J. Mekalanos.** 1999. Genetic characterization of a new type IV-A pilus gene cluster found in both classical and El Tor biotypes of *Vibrio cholerae.* Infect. Immun. **67:**1393–1404.
10. **Hahn, H. P.** 1997. The type-4 pilus is the major virulence-associated adhesin of *Pseudomonas aeruginosa*—a review. Gene **192:**99–108.
11. **Herriott, R. M., E. Y. Meyer, M. Vogt, and M. Modan.** 1970. Defined medium for growth of *Haemophilus influenzae.* J. Bacteriol. **101:**513–516.
12. **Hobbs, M., E. S. Collie, P. D. Free, S. P. Livingston, and J. S. Mattick.** 1993. PilS and PilR, a two-component transcriptional regulatory system controlling expression of type 4 fimbriae in *Pseudomonas aeruginosa.* Mol. Microbiol. **7:**669–682.
13. **Hobbs, M., and J. S. Mattick.** 1993. Common components in the assembly of type 4 fimbriae, DNA transfer systems, filamentous phage and protein-secretion apparatus: a general system for the formation of surface-associated protein complexes. Mol. Microbiol. **10:**233–243.
14. **Hu, N. T., P. F. Lee, and C. Chen.** 1995. The type IV pre-pilin leader peptidase of *Xanthomonas campestris* pv. *campestris* is functional without conserved cysteine residues. Mol. Microbiol. **18:**769–777.
15. **Ishimoto, K. S., and S. Lory.** 1989. Formation of pilin in *Pseudomonas aeruginosa* requires the alternative sigma factor (RpoN) of RNA polymerase. Proc. Natl. Acad. Sci. USA **86:**1954–1957.
16. **Kennan, R. M., O. P. Dhungyel, R. J. Whittington, J. R. Egerton, and J. I. Rood.** 2001. The type IV fimbrial subunit gene (*fimA*) of *Dichelobacter nodosus* is essential for virulence, protease secretion, and natural competence. J. Bacteriol. **183:**4451–4458.
17. **Koga, T., K. Ishimoto, and S. Lory.** 1993. Genetic and functional characterization of the gene cluster specifying expression of *Pseudomonas aeruginosa* pili. Infect. Immun. **61:**1371–1377.
18. **Konings, R. N., E. J. Verhoeven, and B. P. Peeters.** 1987. pKUN, vectors for the separate production of both DNA strands of recombinant plasmids. Methods Enzymol. **153:**12–34.
19. **Koomey, M., S. Bergstrom, M. Blake, and J. Swanson.** 1991. Pilin expression and processing in pilus mutants of *Neisseria gonorrhoeae*: critical role of Gly-1 in assembly. Mol. Microbiol. **5:**279–287.
20. **LaPointe, C. F., and R. K. Taylor.** 2000. The type 4 prepilin peptidases comprise a novel family of aspartic acid proteases. J. Biol. Chem. **275:**1502–1510.
21. **Liles, M. R., V. K. Viswanathan, and N. P. Cianciotto.** 1998. Identification and temperature regulation of *Legionella pneumophila* genes involved in type IV pilus biogenesis and type II protein secretion. Infect. Immun. **66:**1776–1782.
22. **Lory, S., and M. S. Strom.** 1997. Structure-function relationship of type-IV prepilin peptidase of *Pseudomonas aeruginosa*—a review. Gene **192:**117–121.
23. **Mattick, J. S.** 2002. Type IV pili and twitching motility. Annu. Rev. Microbiol. **56:**289–314.
24. **May, B. J., Q. Zhang, L. L. Li, M. L. Paustian, T. S. Whittam, and V. Kapur.** 2001. Complete genomic sequence of *Pasteurella multocida*, Pm70. Proc. Natl. Acad. Sci. USA **98:**3460–3465.
25. **Meier, P., C. Berndt, N. Weger, and W. Wackernagel.** 2002. Natural transformation of *Pseudomonas stutzeri* by single-stranded DNA requires type IV pili, competence state and *comA.* FEMS Microbiol. Lett. **207:**75–80.
26. **Mishra, P., P. K. Park, and D. G. Drueckhammer.** 2001. Identification of *yacE* (*coaE*) as the structural gene for dephosphocoenzyme A kinase in *Escherichia coli* K-12. J. Bacteriol. **183:**2774–2778.
27. **Nassif, X., M. Marceau, C. Pujol, B. Pron, J. L. Beretti, and M. K. Taha.** 1997. Type-4 pili and meningococcal adhesiveness. Gene **192:**149–153.
28. **Paranjpye, R. N., J. C. Lara, J. C. Pepe, C. M. Pepe, and M. S. Strom.** 1998. The type IV leader peptidase/*N*-methyltransferase of *Vibrio vulnificus* controls factors required for adherence to HEp-2 cells and virulence in iron-overloaded mice. Infect. Immun. **66:**5659–5668.
29. **Pepe, C. M., M. W. Eklund, and M. S. Strom.** 1996. Cloning of an *Aeromonas hydrophila* type IV pilus biogenesis gene cluster: complementation of pilus assembly functions and characterization of a type IV leader peptidase/N-methyltransferase required for extracellular protein secretion. Mol. Microbiol. **19:**857–869.
30. **Rossier, O., and N. P. Cianciotto.** 2001. Type II protein secretion is a subset of the PilD-dependent processes that facilitate intracellular infection by *Legionella pneumophila.* Infect. Immun. **69:**2092–2098.
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
32. **Song, Y., and N. J. Sargentini.** 1996. *Escherichia coli* DNA repair genes *radA* and *sms* are the same gene. J. Bacteriol. **178:**5045–5048.
33. **Stevenson, A., J. Macdonald, and M. Roberts.** 2003. Cloning and characterisation of type 4 fimbrial genes from *Actinobacillus pleuropneumoniae.* Vet. Microbiol. **92:**121–134.
34. **Stone, B. J., and Y. Abu Kwaik.** 1998. Expression of multiple pili by *Legionella pneumophila*: identification and characterization of a type IV pilin gene and its role in adherence to mammalian and protozoan cells. Infect. Immun. **66:**1768–1775.
35. **Strom, M. S., P. Bergman, and S. Lory.** 1993. Identification of active-site cysteines in the conserved domain of PilD, the bifunctional type IV pilin leader peptidase/N-methyltransferase of *Pseudomonas aeruginosa.* J. Biol. Chem. **268:**15788–15794.
36. **Strom, M. S., and S. Lory.** 1991. Amino acid substitutions in pilin of *Pseudomonas aeruginosa.* Effect on leader peptide cleavage, amino-terminal methylation, and pilus assembly. J. Biol. Chem. **266:**1656–1664.
37. **Strom, M. S., and S. Lory.** 1993. Structure-function and biogenesis of the type IV pili. Annu. Rev. Microbiol. **47:**565–596.
38. **Strom, M. S., D. N. Nunn, and S. Lory.** 1993. A single bifunctional enzyme, PilD, catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family. Proc. Natl. Acad. Sci. USA **90:**2404–2408.
39. **Taha, M. K., P. C. Morand, Y. Pereira, E. Eugene, D. Giorgini, M. Larribe, and X. Nassif.** 1998. Pilus-mediated adhesion of *Neisseria meningitidis*: the essential role of cell contact-dependent transcriptional upregulation of the PilC1 protein. Mol. Microbiol. **28:**1153–1163.
40. **Tonjum, T., N. E. Freitag, E. Namork, and M. Koomey.** 1995. Identification and characterization of *pilG*, a highly conserved pilus-assembly gene in pathogenic *Neisseria.* Mol. Microbiol. **16:**451–464.
41. **Turner, L. R., J. C. Lara, D. N. Nunn, and S. Lory.** 1993. Mutations in the consensus ATP-binding sites of XcpR and PilB eliminate extracellular protein secretion and pilus biogenesis in *Pseudomonas aeruginosa.* J. Bacteriol. **175:**4962–4969.
42. **Van Leengoed, L. A., and E. M. Kamp.** 1989. Endobronchial inoculation of various doses of *Haemophilus* (*Actinobacillus*) *pleuropneumoniae* in pigs. Am. J. Vet. Res. **50:**2054–2059.
43. **Van Overbeke, I., K. Chiers, G. Charlier, I. Vandenberghe, J. Van Beeumen, R. Ducatelle, and F. Haesebrouck.** 2002. Characterization of the *in vitro* adhesion of *Actinobacillus pleuropneumoniae* to swine alveolar epithelial cells. Vet. Microbiol. **88:**59–74.
44. **Watson, A. A., J. S. Mattick, and R. A. Alm.** 1996. Functional expression of hetereologous type 4 fimbriae in *Pseudomonas aeruginosa.* Gene **175:**143–150.
45. **West, S. E., M. J. Romero, L. B. Regassa, N. A. Zielinski, and R. A. Welch.** 1995. Construction of *Actinobacillus pleuropneumoniae-Escherichia coli* shuttle vectors: expression of antibiotic-resistance genes. Gene **160:**81–86.
45a. **Winther-Larsen, H. C., and M. Koomey.** 2002. Transcriptional, chemosensory and cell-contact-dependent regulation of type IV pilus expression. Curr. Opin. Microbiol. **5:**173–178.
46. **Wolfgang, M., P. Lauer, H. S. Park, L. Brossay, J. Hebert, and M. Koomey.** 1998. PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated *Neisseria gonorrhoeae.* Mol. Microbiol. **29:**321–330.
47. **Wu, H., and P. M. Fives-Taylor.** 2001. Molecular strategies for fimbrial expression and assembly. Crit. Rev. Oral Biol. Med. **12:**101–115.
48. **Zhang, Y., J. M. Tennent, A. Ingham, G. Beddome, C. Prideaux, and W. P. Michalski.** 2000. Identification of type 4 fimbriae in *Actinobacillus pleuropneumoniae.* FEMS Microbiol. Lett. **189:**15–18.

Editor: V. J. DiRita