THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 47, pp. 39104–39114, November 25, 2005

Truncation of the Lipopolysaccharide Outer Core Affects Susceptibility to Antimicrobial Peptides and Virulence of *Actinobacillus pleuropneumoniae* Serotype 1*

Received for publication, March 15, 2005, and in revised form, August 29, 2005 Published, JBC Papers in Press, September 27, 2005, DOI 10.1074/jbc.M502852200

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We reported previously that the core oligosaccharide region of the lipopolysaccharide (LPS) is essential for optimal adhesion of *Actinobacillus pleuropneumoniae*, an important swine pathogen, to respiratory tract cells. Rough LPS and core LPS mutants of *A. pleuropneumoniae* serotype 1 were generated by using a mini-*Tn10* transposon mutagenesis system. Here we performed a structural analysis of the oligosaccharide region of three core LPS mutants that still produce the same O-antigen by using methylation analyses and mass spectrometry. We also performed a kinetic study of proinflammatory cytokines production such as interleukin (IL)-6, tumor necrosis factor-α, IL1-β, MCP-1, and IL8 by LPS-stimulated porcine alveolar macrophages, which showed that purified LPS of the parent strain, the rough LPS and core LPS mutants, had the same ability to stimulate the production of cytokines. Most interestingly, an *in vitro* susceptibility test of these LPS mutants to antimicrobial peptides showed that the three core LPS mutants were more susceptible to cationic peptides than both the rough LPS mutant and the wild type parent strain. Furthermore, experimental pig infections with these mutants revealed that the galactose (Gal I) and D,D-heptose (Hep IV) residues present in the outer core of *A. pleuropneumoniae* serotype 1 LPS are important for adhesion and overall virulence in the natural host, whereas deletion of the terminal GalNAc-Gal II disaccharide had no effect. Our data suggest that an intact core-lipid A region is required for optimal protection of *A. pleuropneumoniae* against cationic peptides and that deletion of specific residues in the outer LPS core results in the attenuation of the virulence of *A. pleuropneumoniae* serotype 1.

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, an infection characterized by hemorrhagic, fibrinous, and necrotic lung lesions. This highly contagious disease is responsible for substantial economic losses in the swine industry (1). The disease may be acute, subacute, or chronic and is normally transmitted by chronically infected pigs. However, it has been reported that pigs may be subclinically infected without presenting clinical signs (2, 3), and these pigs are thought to be the main cause of *A. pleuropneumoniae* dissemination (1). Fifteen serotypes of *A. pleuropneumoniae* based on capsular antigens have been identified, and the most predominant in Quebec, Canada, are serotypes 1, 5, and 7 (4). Among the many virulence factors of *A. pleuropneumoniae*, namely the RTX (repeats-in-toxin) toxins (5), the capsule, and the outer membrane proteins (6–8), the lipopolysaccharides (LPS),[2] are known to be important, because of their involvement in the adhesion to host cells (9, 10), and are also known to play a role in the stimulation of the host immune system. LPS are complex molecules composed of the following three well defined regions: (i) lipid A, anchored in the outer membrane; (ii) the core oligosaccharide containing 2-keto-3-deoxyoctulosonic acid (Kdo) and heptose residues; and (iii) the O-antigen, which is a polysaccharide consisting of repeating units. The structure of *A. pleuropneumoniae* serotype 1 O-antigen has been described as branched tetrasaccharide repeating units composed of two α-L-rhamnopyranosyl, one α-D-glycopyranosyl, and one 2-acetamido-2-deoxy-β-D-glucose residues (11). More recently, the structures of the core oligosaccharide of LPS from *A. pleuropneumoniae* serotypes 1, 2, 5a, and 5b were elucidated using NMR spectroscopy and mass spectrometry (12), which revealed a conserved inner core structure consisting of a trisaccharide of L-*glycero*-D-*manno*-heptose residues linked to a Kdo residue and substituted at different positions as shown previously (12). The study also allowed the identification of a novel open chain GalNAc residue in the outer core of serotype 1 (Fig. 1*A*).

We have previously generated four core LPS and seven rough LPS mutants of *A. pleuropneumoniae* serotype 1 by using a mini-*Tn10* mutagenesis system, and the gene affected by the transposon was identified in each of the mutants (13–15). Characterization of the rough LPS mutants allowed us to identify a cluster of 13 genes involved in O-polysaccharide biosynthesis in *A. pleuropneumoniae* serotype 1 (15). The genes affected in the core LPS mutants were *galU* in mutant 5.1 (13), which encodes a UTP-α-D-glucose-1-phosphate uridylyltransferase, a gene involved in outer core elongation with galactose in mutant CG1 (14) and a gene coding for a D-*glycero*-D-*manno*-heptosyltransferase in mutants CG3 and CG5 (14). All these core LPS mutants still express an O-chain, although their core oligosaccharide is apparently truncated,

* This work was supported in part by the Natural Sciences and Engineering Research Council of Canada Grant DGPIN3428 (to M. J.) and Fonds pour la Formation de Chercheurs et l'Aide à la Recherche Grant 2002-ER-71900. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: LPS, lipopolysaccharide; CE-ESIMS, capillary electrophoresis-electrospray ionization mass spectrometry; MIC, minimum inhibitory concentration; IL, interleukin; TNF, tumor necrosis factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAMs, porcine alveolar macrophages; DMEM, Dulbecco's modified Eagle's medium; CFU, colony-forming units; RT, reverse transcriptase; Kdo, 2-keto-3-deoxyoctulosonic acid; Hep, heptose; Hex, hexose; PMAA, partially methylated alditol acetates; CE-MS, capillary electrophoresis mass spectrometry; ELISA, enzyme-linked immunosorbent assays;PEA, phosphorylethanolamine.

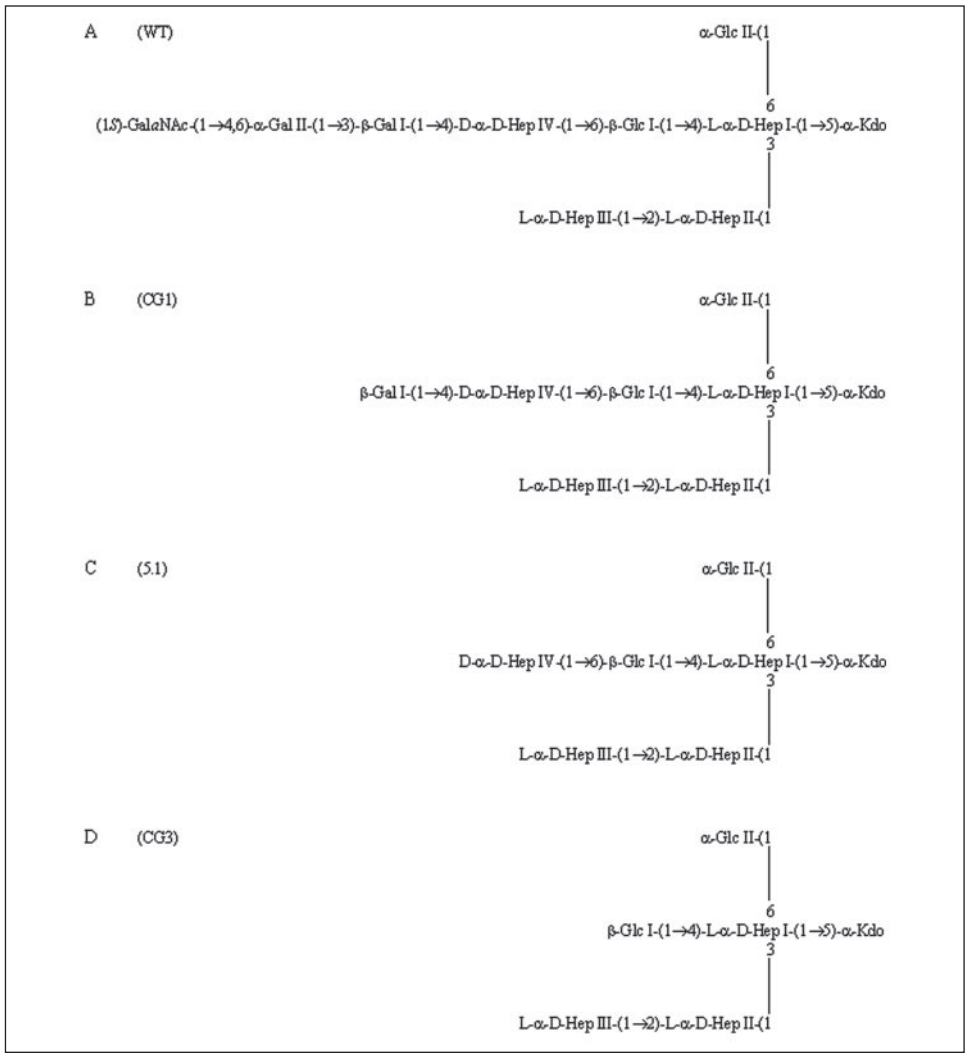

FIGURE 1. **Structural representation of the core oligosaccharide from *A. pleuropneumoniae* serotype 1 parent strain (*A*), the core LPS mutant CG1 (*B*), the core LPS mutant 5.1 (*C*), and the core LPS mutant CG3 (*D*).**

based on gel mobility (14). Characterization of the surface properties of these LPS mutants demonstrated that O-antigen deletions in rough mutants have no effect on the adhesion of the bacteria to frozen tracheal sections of pigs, whereas an intact core oligosaccharide region seems to be required for optimal adherence (7, 13). Most interestingly, *in vivo* experiments in pigs showed that the core LPS mutant 5.1 that still produces an O-antigen was less virulent, whereas no difference was observed between the rough mutant 27.1 lacking O-antigen and the parent strain (13–15). These observations suggest that the LPS core oligosaccharide could play a major role in colonization and pathogenesis of *A. pleuropneumoniae* in pigs.

Another important feature of LPS molecules is their interaction with the host immune system. The innate immunity is the first barrier for invading organisms, and inflammation is known to be of special importance in this first line of defense (16). Porcine pleuropneumonia is characterized by an intensive inflammation with infiltration of phagocytic cells such as neutrophils and alveolar macrophages, which are the main cells found in affected lungs (17). Previous *in vivo* studies have shown that *A. pleuropneumoniae* infection is associated with the production of a large amount of inflammatory mediators (18, 19) and proinflammatory cytokines such as IL1, TNF-α, IL8, and IL6 (20, 21) that could be responsible, at least in part, for the lung injuries and tissue destruction observed in porcine pleuropneumonia (20, 22). LPS are known as mediators of inflammation (16) as they interact with immune cells and activate the production of inflammatory cytokines (23, 24) by these cells. Thus, by interacting with the immune system and promoting inflammation, LPS could be an important factor in the development of lesions observed in porcine pleuropneumonia. We sought to examine if a defined mutation within the LPS could result in a decrease in LPS-induced cytokine production and consequently cause an attenuation of the virulence of *A. pleuropneumoniae*. One other important component of the innate immune system is antimicrobial peptides, which can either be released by epithelial cells or by phagocytic cells (25). Most of these peptides are small cationic peptides that have been isolated from various biological sources (26); they act mainly by forming pores in bacterial membranes (27). They are classified into the following three major classes on the basis of their amino acid compositions and their three-dimensional structures: linear α-helical peptides without cysteines (cecropin from pigs and magainin from frogs); peptides with a predominantly β-sheet structure with cysteines linked by disulfide bridges (defensin and protegrin from pigs); and peptides with high contents of particular amino acids (PR-39 from pigs) (28). As a result of their positive charges, these peptides have a high affinity for negatively charged LPS that form the external leaflet of the outer membrane of Gram-negative bacteria. Thus, a modification within these LPS molecules could result in an increase of susceptibility to these peptides.

Considering the potential role played by the LPS core oligosaccharide in *A. pleuropneumoniae* pathogenesis, the purpose of the present study

TABLE ONE

Bacterial strains used in the present study

Strains	Relevant traits	Source or Ref.
A. pleuropneumoniae[a] S4074 Nal^r	Serotype 1 (Nal^r), parent strain	13
27.1[b]	LPS O-antigen mutant	13
5.1[b]	LPS core oligosaccharide mutant	13
CG1[b]	LPS core oligosaccharide mutant	14
CG3[b]	LPS core oligosaccharide mutant	14
33.2[b]	Acapsular mutant	34
E. coli K88ac	K12 with fimbriae F4ac	Our collection

[a] The nalidixic acid-resistant (Nal^r) strain was obtained from the reference strain *A. pleuropneumoniae* S4074 serotype 1 (K. R. Mittal, Faculté de Médecine Vétérinaire, Université de Montréal).
[b] All the mutants were derived from *A. pleuropneumoniae* serotype 1 S4074 Nal^r.

was to determine the core oligosaccharide structure of three different core LPS mutants of *A. pleuropneumoniae* serotype 1 (5.1, CG1, and CG3), by using capillary electrophoresis-electrospray ionization mass spectrometry (CE-ESI/MS) in combination with methylation analysis, and to compare those structures to that of the wild type parent strain. To consider the effect of these mutations on the interaction of *A. pleuropneumoniae* with immune system components, a kinetic study of pro-inflammatory cytokines production was performed on porcine alveolar macrophages stimulated with either the LPS mutants or the wild type parent strain. The susceptibility of the core LPS mutants to antimicrobial peptides was also evaluated by using an *in vitro* assay to determine the minimum inhibitory concentration (MIC) of various cationic peptides. Finally, experimental pig infections were performed with these core LPS mutants.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions—The bacterial strains used in this study are shown in TABLE ONE. *A. pleuropneumoniae* S4074 Nal^r was grown on BHI (Difco) agar plates supplemented with 15 μg of NAD per ml and 30 μg of nalidixic acid (Nal) per ml. Transpositional mutants (TABLE ONE) were grown on BHI agar plates supplemented with 15 μg/ml NAD, 30 μg/ml nalidixic acid, and 75 μg/ml kanamycin. *Escherichia coli* K88ac was grown on LB (Difco) agar plates. Liquid cultures of *A. pleuropneumoniae* strains (wild type and mutants) were done in BHI broth supplemented with 5 μg/ml NAD, whereas the *E. coli* strain was grown in LB broth.

Isolation of LPS—The LPS was isolated as described previously (12), giving 110 mg for mutant 5.1, 44 mg for mutant CG1, and 35 mg for mutant CG3. 36 mg of mutant 5.1 LPS, 30 mg of mutant CG1 LPS, and 35 mg (all but 0.2 mg used) of mutant CG3 LPS were each purified down a column of Bio-Gel P-2 and eluted with water as described previously (12).

Deacylation of LPS— 0.2 mg of LPS of each mutant was *O*-deacylated as described previously (12) and examined by CE-MS.

Isolation of the Core Oligosaccharide—The sugar-containing fractions of each mutant LPS following column chromatography were pooled and treated with 3.5 ml of 2% acetic acid at 100 °C for 2 h, giving core oligosaccharide (19.3 mg for mutant 5.1, 15.6 mg for mutant CG1, and 21.3 mg for mutant CG3). The samples were purified on a Bio-Gel P-2 column as described previously (12). Core fractions (fractions 19–24) eluting off the column slightly after the O-chain (fractions 15–17) were combined and lyophilized.

Microanalysis of Cells— 0.2 mg of cells of each mutant was suspended in 200 μl of water, and 2 μl of 1 mg/ml proteinase K was added. The samples were left at 37 °C for 5 h and then heated to 75 °C for 10 min (to destroy the enzyme) and lyophilized. The samples were resuspended in 200 μl of 20 mM ammonium acetate buffer (pH 7.5), and 1 μl of DNase and 2 μl of RNase (both 10 mg/ml) was added and left at 37 °C for 6 h and lyophilized. The samples were then deacylated as described above for purified LPS and sent for CE-MS.

Analytical Methods and Mass Spectrometry—The structural analysis was performed as described previously (12).

Isolation of Porcine Alveolar Macrophages (PAMs)—PAMs were obtained from two healthy pigs that originated from herds known to be serologically free for all serotypes of *A. pleuropneumoniae*, by means of lung lavage as described previously (29). Briefly, the pigs were pre-anesthetized through an intramuscular injection of 22 mg/kg ketamine and 4 mg/kg stresnil and euthanized intravenously with 60 mg/kg pentobarbital. Three bronchoalveolar lavages were performed for each pig with a total of 300 ml of sterile pyrogen-free phosphate-buffered saline per lavage. The fluid lavage was centrifuged at 800 $\times$ *g* for 10 min, and the cell pellets were washed and resuspended in DMEM (12430-054; Invitrogen) supplemented with 40% fetal bovine serum (Invitrogen). The cells were cultured in 24-well tissue culture plates (BD Biosciences), and nonadherent cells were removed by washing the plates three times with phosphate-buffered saline. The cell concentration was then adjusted to 2×10^7/ml in a medium containing 80% DMEM and 20% Me_2SO and cryopreserved in liquid nitrogen until used.

Stimulation of Cytokine Production—Before the experiment, PAMs were quickly thawed in a water bath at 37 °C, washed, and resuspended in complete DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin, 0.1 mM minimum Eagle's medium, nonessential amino acids (all purchased from Invitrogen) and 1 mM sodium pyruvate (Sigma). Cell count was re-evaluated on the basis of trypan blue dye exclusion, and the cell concentration was adjusted to 1×10^6/ml. PAMs were then dispensed in 24-well tissue culture plates and incubated overnight at 37 °C in 5% CO_2. For the kinetic study the PAMs were classified into the following three groups: unstimulated cells as a negative control, cells stimulated with 1 μg/ml purified LPS, or 10^9 CFU/ml of heat-killed bacteria and incubated at 37 °C in 5% CO_2. Purification of LPS was performed according to the Darveau-Hancock procedure (30, 31), and the products were separated and analyzed by SDS-PAGE and immunoblotting as described previously (13). Purified LPS of the wild type parent strain and all the LPS mutants (TABLE ONE) were used in this study. As a positive control of the stimulation, purified LPS of *E. coli* 0127:B8 (Sigma) was also used. Heat-killed bacteria from all the *A. pleuropneumoniae* strains shown in TABLE ONE were also used in this study and prepared as described previously (32) by incubating the organisms at 60 °C for 45 min. Stimulated or unstimulated (control) samples were collected at different time intervals (0.5, 1, 2, 4, 6, 12, 24, and 48 h). At each sampling, cell-free supernatants were collected, ali-

TABLE TWO

Nucleotide sequences of the primer sets used to amplify porcine cytokine genes

The size of the PCR products are represented. For indicates forward; Rev indicates reverse.

Gene	Oligonucleotide sequence		PCR product
			bp
TNF-α	For	5′-CAC TGA GAG CAT GAT CCG AG-3′	470
	Rev	5′-GGC TGA TGG TGT GAG TGA GG-3′	
IL1-β	For	5′-TCA GGC AGA TGG TGT TCT GTC-3′	430
	Rev	5′-GGT CTA TAT CCT CCA GCT GC-3′	
IL6	For	5′-GGA ACG CCT GGA AGA AGA TG-3′	470
	Rev	5′-ATC CAC TCG TTC TGT GAC TG-3′	
MCP-1	For	5′-ATT AAT TCT CCA GTC ACC TG-3′	420
	Rev	5′-AAC ACC AGT AGT CAT GGA GG-3′	
IL8	For	5′-TGC AGC TTC ATG GAC CAG-3′	350
	Rev	5′-TGT TGC TTC TCA GTT CTC TTC-3′	
GAPDH	For	5′-CAC TGG TGT CTT CAC GAC-3′	295
	Rev	5′-GCC ATC CAC AGT CTT CTG-3′	
B2M	For	5′-CTG CTC TCA CTG TCT GG-3′	295
	Rev	5′-ATC GAG AGT CAC GTG CT-3′	

quoted, and stored at −20 °C for ELISA cytokine quantification. Cells were then treated for total RNA extraction for RT-PCR analysis of cytokine gene expression. As a control of the LPS-stimulated cytokines production, a 24-h incubation time of PAMs with purified LPS was carried out as described above in the presence of 10 μg/ml polymyxin B (Sigma), an LPS inhibitor. A cytotoxicity test based on lactate dehydrogenase dosage in the supernatants showed that neither the purified LPS nor the heat-killed bacteria have a cytotoxic effect on the PAMs during the incubation times (data not shown). This test was performed using a CytoTox 96 LDH kit (Promega, Madison, WI) according to the manufacturer's protocol.

Enzyme-linked Immunosorbent Assays (ELISA) for Cytokines—IL1-β was quantified using the immunoassay kit swine IL1-β (BIOSOURCE), as specified by the manufacturer. TNF-α, IL-6, and IL-8 were measured by sandwich ELISA, using the following pair-matched monoclonal antibodies from R & D Systems (Minneapolis, MN), according to the manufacturer's recommendations: TNF-α, monoclonal anti-porcine TNF-α and biotinylated anti-porcine TNF-α; IL-6, polyclonal anti-porcine IL-6 and biotinylated anti-porcine IL-6; and IL-8, monoclonal anti-human IL-8 and biotinylated anti-porcine IL-8. 2-Fold dilutions of recombinant porcine TNF-α, IL-6 (78–5000 pg/ml; R & D Systems), and porcine IL-8 (18–600 pg/ml; R & D Systems) were included as standard curves in each ELISA plate (Nunc, VWR, Ville Mont Royal, Quebec, Canada). Sample dilutions giving optical density readings in the linear portion of the appropriate standard curve were used to quantify the levels of each cytokine in the samples. Standard and sample dilutions were added in duplicate wells to each ELISA plate, and all analyses were performed at least four times for each individual stimulation assay. Plates were read in a Power Wave X 340 (Biotek Instruments Inc., Winooski, VT) microplate reader.

Total RNA Extraction—Macrophages were resuspended and lysed by repeated pipetting in 1 ml of Trizol reagent (Invitrogen). Total RNA was extracted as specified by the manufacturer. The final RNA pellet was resuspended in 25 μl of diethyl pyrocarbonate-treated water, and RNA concentration and purity were measured using an Ultrospec 2100 pro-UV-visible spectrophotometer (Biochrom Ltd., Cambridge, UK). A PCR using primers for a constitutive gene, β_2-microglobulin (TABLE TWO), was performed with the RNA samples, as described below, to detect any DNA contamination. RNA was stored at −80 °C for future use.

Reverse Transcriptase-PCR for Porcine Cytokines—Reverse transcription of RNA into cDNA was performed in a 40-μl total volume containing 1 μg of sample RNA, 400 ng of random primers (Roche Diagnostics), 0.5 mM dNTP mix (Amersham Biosciences), 1× first-strand buffer, 10 mM dithiothreitol, 80 units of RNAguard RNase inhibitor (Amersham Biosciences), 400 units of Superscript II RNaseH reverse transcriptase (Invitrogen). Briefly template RNA, dNTPs, and random primers were incubated at 65 °C for 5 min followed by a step at 4 °C. All components except the reverse transcriptase were then added, and the sample was incubated at 25 °C for 10 min and 42 °C for 2 min. Superscript reverse transcriptase was finally added, and the sample was incubated for 50 min at 42 °C. A final heating step at 70 °C for 15 min was also performed for enzyme inactivation. The newly synthesized cDNA samples were stored at −20 °C until used for PCRs.

The oligonucleotide primers used for the detection of cDNA specific to porcine TNF-α, IL1-β, IL6, MCP-1, IL 8, and primers for control housekeeping genes β_2-microglobulin (β2-M) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are represented in TABLE TWO. It is worth noting that the cytokine fragment and the appropriate constitutive gene fragment (TABLE TWO) were amplified in the same reaction to minimize the risk of variations. The PCR mixtures for amplification of cDNA were performed in a 25-μl total volume containing 0.4 mM dNTP mix (Amersham Biosciences), 1× PCR buffer, 0.16 mM forward and reverse primers for the cytokine and the housekeeping gene, 1 μl of cDNA template, and 2.5 units of *Taq* polymerase (Amersham Biosciences). The PCR was performed in a Biometra *Tpersonal* thermocycler as follows. Initial denaturation was for 3 min at 94 °C followed by 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and elongation for 30 s at 72 °C. The reaction was completed with a final elongation step at 72 °C for 7 min. A negative PCR control (all components except cDNA) was included in all PCRs. 10 μl of the PCR products were separated by electrophoresis in 2% agarose gel with ethidium bromide, visualized in a MultiImage Light Cabinet apparatus, and band intensity was determined by use of an Alpha Imager 2000 software (Alpha Innotech Corp.). Correct size was verified by comparison with a 100-bp ladder (Roche Diagnostics). The relative band intensity was used to determine the cytokine/housekeeping gene ratio expressed as arbitrary units.

In Vitro Susceptibility Test—One or two colonies of the strains to be tested were grown in 5 ml of broth with shaking at 150 rpm for 4 h to an

TABLE THREE

Overview of antimicrobial peptides used in this study

Peptide	Sequence	Structure	Origin
Magainin-1	GIGKFLHSAGKFGKAFVGZIMKS	α-Helix	Frog
Melittin	GIGAILKVLATGLPTLISWIKNKRKQ	α-Helix	Honey bee
Mastoparan	VDWKKIGQHILSVL	α-Helix	*Polistes jadwigae*
Cecropin P1	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR	α-Helix	Pig
Protegrin-1	RGGRLCYCRRRFCVCVGR	β-Sheet	Pig
Indolicidin	ILPWKWPWWPWRR	Linear	Cow

$A_{600\ nm}$ of 0.7. The cultures were then diluted in fresh media to 5×10^5 CFU/ml and used as the inoculum for the susceptibility test. Polymyxin B, protamine, cecropin P1, melittin, mastoparan (all from Sigma), and protegrin-1 (provided by R. I. Lehrer, UCLA School of Medicine) were assayed at the following final concentrations: 0.8–100 μg/ml for melittin, mastoparan, and protegrin; 0.08–10 μg/ml for cecropin P1; 0.01 to 2 μg/ml for polymyxin B; and 8–1000 μg/ml for protamine. Magainin-1, indolicidin, and bacitracin, all purchased from Sigma, were also used in this study (TABLE THREE). Serial dilutions of the peptides were prepared in sterile water, and 10 μl of the dilutions were dispensed in duplicate in sterile 96-well polystyrene microtiter plates (Corning Inc.); 90 μl of the bacterial inoculum was then added to each peptide-containing well, and the mixture was incubated at 37 °C. The MIC was determined as the lowest concentration of the compound that did not allow visible growth after 18–24 h of incubation. Growth was evaluated visually and also measured at 620 nm with a Dynatech MR5000 microplate reader (Dynatech Laboratories Inc., Chantilly, VA). As controls, wells without peptides or without bacteria were carried out with each experiment. An *E. coli* K88ac strain was used as a control for the antimicrobial activity of the peptides.

Experimental Pig Infection—Fourteen specific pathogen-free 9-week-old piglets (AFSSA Ploufragan, France) were used and separated into two groups of five pigs, and one group of four pigs housed separately in a pathogen-free environment. The pigs were infected at 12 weeks of age; one group received the parent strain S4074 Nal^r, one group received the LPS core mutant CG1, and the last group received the LPS core mutant CG3. Pigs were challenged once intranasally with a total of 1 ml (0.5 ml per nostril) of a 6-h culture containing ~1×10^6 CFU. Animals were monitored daily for fever, cough, dyspnea, and anorexia; blood samples were also collected weekly for serum analysis using an immunoenzymatic ELISA (Swinecheck App 1, 9, 11, Biovet, St.-Hyacinthe, Quebec, Canada) for the detection of antibodies against LPS of *A. pleuropneumoniae* serotype 1. The performances of the animals (daily average weight gain) during the experiment were also evaluated. Pigs were euthanized 15 days after infection, and necropsy was carried out with every pig for macroscopic and microscopic examination of lesions within the lungs. For histological preparation, lung samples were fixed in 10% buffered formalin and embedded in paraffin, and 6-μm-thick sections were cut and stained with hematoxylin, phloxin, and saffron. Lungs, tonsils, and nasal cavities were cultured for reisolation of challenge bacteria using trypticase soy agar (Difco) enriched with 1 μg/ml crystal violet, 1 μg/ml lincomycin, 128 μg/ml bacitracin, 1 mg/ml NAD, 5% (v/v) sheep blood, and 5% (w/v) yeast extract. Isolates were inoculated on selective media described above to confirm that the mutant strains or the parent strain used for challenge were indeed isolated. PCR detection of the bacteria based on the amplification of the *omlA* gene, coding for an outer membrane protein of *A. pleuropneumoniae*, was also performed with lung samples (33).

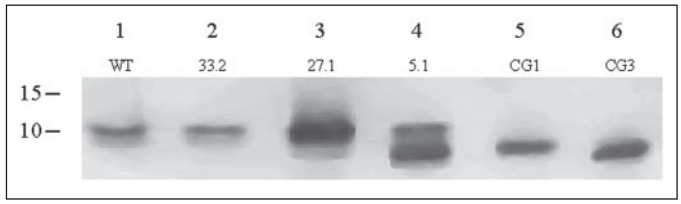

FIGURE 2. **Silver-stained SDS-PAGE profile of purified LPS from *A. pleuropneumoniae* parent strain 4074 Nal^r and LPS mutants.** *Lane 1,* parent strain; *lane 2*, acapsular mutant 33.2; *lane 3*, rough LPS mutant 27.1; *lane 4*, core LPS mutant 5.1; *lane 5*, core LPS mutant CG1; *lane 6*, core LPS mutant CG3. This gel shows the difference of migration of the core-lipid A between the wild type (*WT*) parent strain and the LPS mutants. Molecular mass markers (in kilodaltons) are indicated on the *left*.

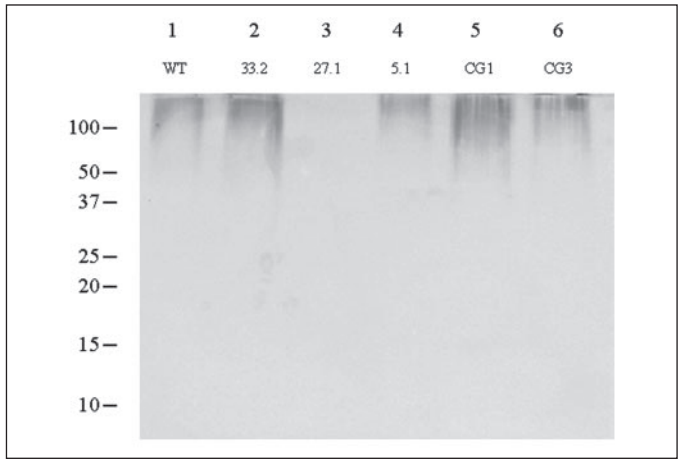

FIGURE 3. **Immunoblot of purified LPS from *A. pleuropneumoniae* parent strain 4074 Nal^r, acapsular mutant, and LPS mutants.** The immunoblot was probed with monoclonal antibody 5.1 G8 F10 against *A. pleuropneumoniae* serotype 1 O-antigen. *Lane 1*, parent strain; *lane 2*, acapsular mutant 33.2; *lane 3*, rough LPS mutant 27.1; *lane 4*, core LPS mutant 5.1; *lane 5*, core LPS mutant CG1; *lane 6*, core LPS mutant CG3. *WT*, wild type. Molecular mass markers (in kilodaltons) are indicated on the *left*.

RESULTS

Structural Analysis of the Core Oligosaccharide of the Three A. pleuropneumoniae Core LPS Mutants—Silver-stained SDS gels of purified LPS of the wild type parent strain and the LPS mutants (Fig. 2) confirmed the differences observed previously in the migration of low molecular mass bands corresponding to the core-lipid A region (13, 14). The rough LPS mutant 27.1 (Fig. 2, *lane 3*) had the same mobility as the parent strain (*lane 1*). The core LPS mutant 5.1 showed an additional band of lower molecular mass (Fig. 2, *lane 4*). The core oligosaccharide region of mutants CG1 (Fig. 2, *lane 5*) and CG3 (*lane 6*) migrated faster than that of the wild type parent strain, with a faster migration for mutant CG3. The parent strain and the acapsular mutant 33.2 (Fig. 2, *lane 2*) showed similar migration profiles as they elaborate the same LPS (34). Western blot analysis using the monoclonal antibody 5.1 G8 F10 directed against the O-antigen of *A. pleuropneumoniae* serotype 1 (Fig.

TABLE FOUR

Negative ion CE-ESIMS data and proposed compositions of O-deacylated LPS and core oligosaccharides from *A. pleuropneumoniae* serotype 1 parent strain and mutants CG1, 5.1, and CG3

Average mass units were used for calculation of molecular weight based on proposed composition as follows: Hex, 162.15; HexNAc, 203.19; Hep, 192.17; Kdo, 220.18; *P*Etn,123.05; *P*, 79.98. The average molecular weight of *O*-deacylated (*O*-deac) lipid A (lipid A-OH) is 952.00. Data acquired by CE-ESIMS on a crystal model 310 CE instrument interfaced to an API 3000 triple quadrupole mass spectrometer (PerkinElmer Life Science/Sciex) fitted with a bare fused silica capillary column and using 30 mM morpholine acetate (pH 9.0) containing 5% methanol as the separation buffer. Tr, trace; *P*Etn, phosphorylethanolamine; *P*, phosphate.

Strain	Observed ions (*m/z*)		Molecular mass (Da)		Relative	Proposed composition
	$(M - 2H)^{2-}$	$(M - 3H)^{3-}$	Observed	Calculated	intensity[a]	
Parent	1396	930	2794	2792.65	0.4	HexNAc, 4Hex, 4Hep, Kdo, Lipid A-OH
O-Deac	1436	957	2874	2872.63	1.0	HexNAc, 4Hex, 4Hep, Kdo, Lipid A-OH, *P*
	1497	998	2996	2995.68	0.75	HexNAc, 4Hex, 4Hep, Kdo, Lipid A-OH, *P*, *P*Etn
CG1	1253	835	2508	2507.29	0.55	3Hex, 4Hep, Kdo, Lipid A-OH, *P*
O-Deac	1315	876	2632	2630.34	1	3Hex, 4Hep, Kdo, Lipid A-OH, *P*, *P*Etn
5.1	1172	781	2346	2345.14	1	2Hex, 4Hep, Kdo, Lipid A-OH, *P*
O-Deac	1234	822	2470	2468.19	0.5	2Hex, 4Hep, Kdo, Lipid A-OH, *P*, *P*Etn
	1253	835	2508	2507.29	0.9	3Hex, 4Hep, Kdo, Lipid A-OH, *P*
	1315	876	2632	2630.34	0.75	3Hex, 4Hep, Kdo, Lipid A-OH, *P*, *P*Etn
	1335	899	2672	2669.44	0.85	4Hex, 4Hep, Kdo, Lipid A-OH, *P*
	1355	903	2714	2710.48	0.3	HexNAc, 3Hex, 4Hep, Kdo, Lipid A-OH, *P*
	1436	957	2874	2872.63	0.85	HexNAc, 4Hex, 4Hep, Kdo, Lipid A-OH, *P*
	1497	998	2996	2995.68	0.6	HexNAc, 4Hex, 4Hep, Kdo, Lipid A-OH, *P*, *P*Etn
CG3	1076	717	2154	2152.97	0.8	2Hex, 3Hep, Kdo, Lipid A-OH, *P*
O-Deac	1138	758	2278	2276.02	1.0	2Hex, 3Hep, Kdo, Lipid A-OH, *P*, *P*Etn
	1157		2316	2315.12	Tr	3Hex, 3Hep, Kdo, Lipid A-OH, *P*
	1200		2402	2399.07	0.5	2Hex, 3Hep, Kdo, Lipid A-OH, *P*, 2*P*Etn
Parent	919		1840	1840.65	1	HexNAc, 4Hex, 4Hep, Kdo
Core OS	958		1919	1820.63	0.05	HexNAc, 4Hex, 4Hep, Kdo, *P*
CG1	560		1120	1120.99	Tr	2Hex, 3Hep, Kdo
Core OS	656		1312	1313.16	0.25	2Hex, 4Hep, Kdo
	737		1474	1475.31	1	3Hex, 4Hep, Kdo
5.1	573		1150	1150.51	0.7	Hex, 4Hep, Kdo
Core OS	655		1312	1312.66	1	2Hex, 4Hep, Kdo
	737		1474	1475.31	0.5	3Hex, 4Hep, Kdo
	838		1678	1678.50	0.1	HexNAc, 3Hex, 4Hep, Kdo
CG3	560		1120	1120.99	1	2Hex, 3Hep, Kdo
Core OS						

[a] Intensity was measured relative to the largest peak in the triply charged region.

3) showed clearly that purified LPS from the three core LPS mutants 5.1, CG1, and CG3 still have the high molecular mass O-chains (Fig. 3, *lanes 4–6*). It is also interesting to note that a comparable amount of these O-chains is observed in purified LPS from the parent strain (Fig. 3, *lane 1*). The rough LPS mutant 27.1 showed no reactivity with the monoclonal antibody (Fig. 3, *lane 3*) because it lacks the O-antigen. As mentioned above, the acapsular mutant 33.2 and the parent strain generate the same LPS. As a result, purified LPS from these two strains contain the O-antigen (Fig. 3, *lanes 1* and *2*).

As reported recently (12), MS of the *O*-deacylated LPS sample of *A. pleuropneumoniae* serotype 1 gave three peaks at $m/z = 2794$ (lipid A, 1Kdo, 4Hep, 4Hex, and HexNAc), $m/z = 2874$ (+PO_4), and $m/z = 2996$ (+PEA and +PO_4), whereas the core oligosaccharide of the parent strain MS gave a mass of 1840 that corresponds to Kdo, 4Hep, 4Hex, and HexNAc (TABLE FOUR and Fig. 1*A*). The sugar analysis of the alditol acetates sample revealed the presence of Glc, Gal, D,D-Hep, and LD-Hep in the ratio 2:1.5:1:2:0. In order to determine the linkage pattern of the molecule, partially methylated alditol acetates (PMAA) analysis by gas chromatography-mass spectrometry was carried out; the sugars obtained were terminal Glc, 6-Glc, 3-Gal, terminal LD-Hep, 4/6-Gal, 4-D,D-Hep, 2-LD-Hep, and 3/4/6-Hep in the ratio of 1:1.5:1:0.5:1:1:1:1 and the proposed structure is shown in Fig. 1*A*. Further sophisticated NMR studies had also been used to identify the nature of the HexNAc, which was found to be a novel open chain GalNAc (12).

In the present study, the structural analysis of the three core LPS mutants was investigated using CE-ESIMS in combination with methylation analysis and compared with the parent strain. The MS of the deacylated LPS sample of the mutant CG1 gave two peaks at $m/z = 2508$ (lipid A, 1Kdo, 1PO_4, 4Hep, and 3Hex) and $m/z = 2632$ (+PEA). The major peak in the core was $m/z = 1474$ (1Kdo, 4Hep, and 3Hex), but there were minor amounts of $m/z = 1312$ (−1Hex) and 1120 (−Hex and Hep) (TABLE FOUR). The alditol acetates sample contained 1 Glc, 1.2 Gal, 0.5 D,D-Hep, and 0.5 LD-Hep, and the PMAA analysis revealed *t*-Glc, 6-Hex, *t*-Hep, 4-Hep, 2-Hep, and 3/4/6-Hep in the ratio of 1:1.5:0.1:0.5:0.5:1. Compared with the parent structure, this mutation has effected the addition of the terminal GalNAc-Gal II disaccharide (Fig. 1*B*), which is in agreement with the insertion of the mini-*Tn10* in *wlaC*, a gene involved in outer core elongation with galactose (14).

MS of the deacylated LPS of the mutant 5.1 gave $m/z = 2346$ as the most intense peak, and this corresponds to a structure of lipid A, 1Kdo, 1PO_4, 4Hep, and 2Hex. There were also peaks at 2470 (+PEA), 2508 (+Hex), 2632(+Hex and PEA), 2672 (+2Hex), 2714 (+Hex and HexNAc, small peak), 2874 (+2Hex and HexNAc), and 2996 (+2Hex, HexNAc, and PEA) (TABLE FOUR). The core MS gave $m/z = 1150$ (1Kdo,

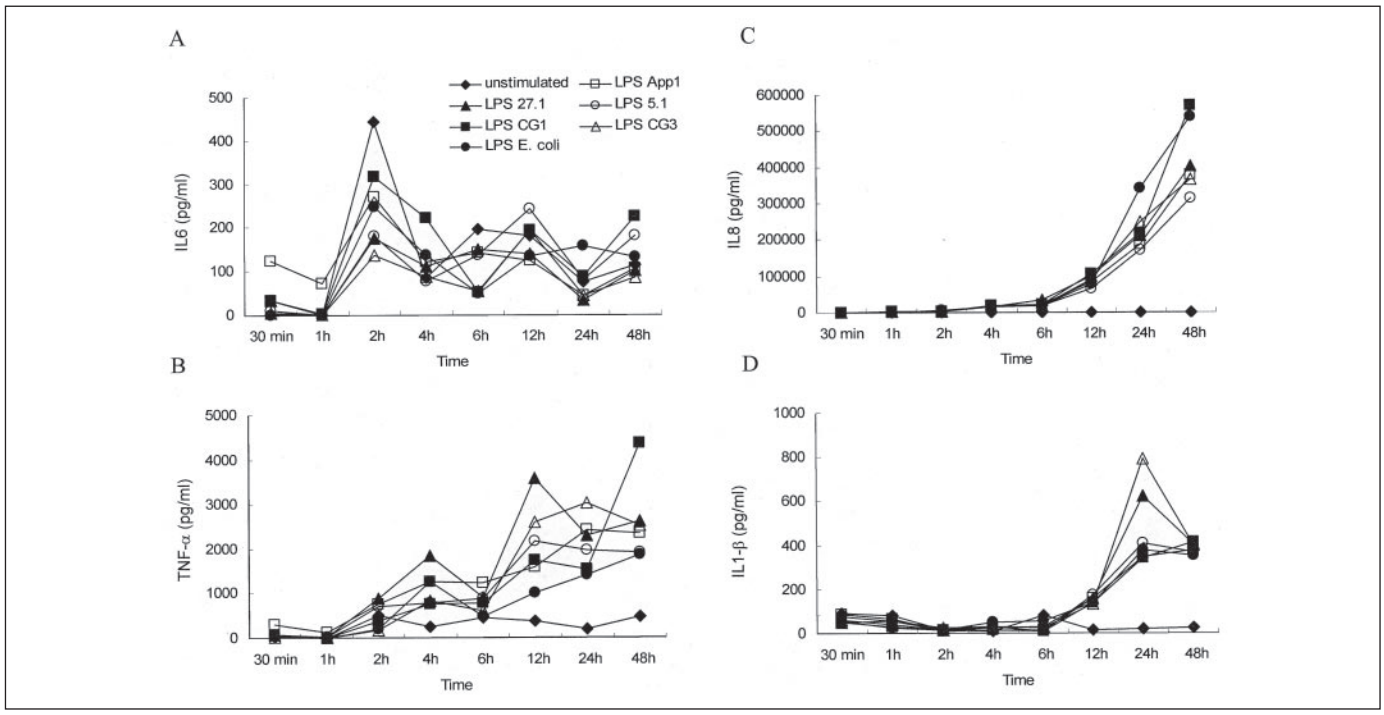

FIGURE 4. **Time course of production of IL6 (*A*), TNF-α (*B*), IL8 (*C*), and IL1-β (*D*) by unstimulated and stimulated PAMs with 1 μg/ml purified LPS from the wild type parent strain *A. pleuropneumoniae* serotype 1, the LPS mutants, and *E. coli* (used as a positive control).** Culture supernatant fluids were collected at different time intervals and assayed for cytokine production by ELISA. Data are expressed in pg/ml.

4Hep, 1Hex), 1312 (+Hex), 1474 (+2Hex), and 1678 (+2Hex and HexNAc) (TABLE FOUR). The alditol acetates analysis revealed 2 Glc:2.1 Gal:1 D,D-Hep:2 L,D-Hep, and the PMAA showed the presence of *t*-Glc, 6-Hex, 3-Hex, *t*-Hep, 4/6-Hex, 4-Hep, 2-Hep, and 3/4/6-Hep in a ratio of 1:1:0.25:0.1:0.25:0.25:0.5:0.5. The most prominent and truncated glycoform of this mutant contains one less Hex residue than mutant CG1 and compared with the parental structure lacks both GalNAc-Gal II and Gal I (Fig. 1*C*). This major truncated glycoform is in agreement with the mutation of *galU* involved in the synthesis of UDP-glucose (13) and with the faster migrating low molecular mass band observed in SDS-PAGE profile of core LPS mutant 5.1 (Fig. 2, *lane 4*). However, the structural data (TABLE FOUR) also showed that this mutant produces small amounts of a full sized core-lipid A that migrated with the same mobility as the parent strain (Fig. 2, *lane 4*), as glycoforms consistent with extension beyond this Gal I residue are still observed.

MS of the deacylated LPS sample of the mutant CG3 gave several peaks all based on the m/z = 2154 (lipid A, 1Kdo, 1PO_4, 3Hep, and 2Hex). The others were m/z = 2278 (+PEA), 2316 (+Hex), and 2402 (+2PEA). The core only contained m/z = 1120 (1Kdo, 3Hep, and 2Hex) (TABLE FOUR). The alditol acetates sample had 1 Glc:0.5 Gal:0.1 D,D-Hep:1 L,D-Hep, and the PMAA contained *t*-Glc, 6-Hex, *t*-Hep, 2-Hep, and 3/4/6-Hep in the ratio of 2:0.1:0.25:1:1. In this mutant we see the loss of one Hep compared with the most truncated and common glycoform of the 5.1 mutant, and this is reflected in the alditol acetates analysis by the loss of most of the D,D-Hep. These data suggest that the tri-L,D-heptosyl inner core is intact, whereas the D,D-Hep from the extension of Hep I has been lost (Fig. 1*D*). Again, this is in agreement with the insertion of the mini-*Tn10* in *lbgB*, a gene coding for a heptosyltransferase (14).

It is also worth noting that the O-chain is still attached in all three core LPS mutants as evidenced by Western blots with O-chain-specific antibodies (Fig. 3), sugar analysis of nonfractionated LPS, and elution profile of the core hydrolysate during column chromatography (data not shown), suggesting that the point of attachment of the O-chain has not been compromised by the different mutations.

Kinetic of Cytokines Production—The data obtained from the kinetic study (Figs. 4 and 5) showed that incubation of PAMs with purified LPS resulted in an increase in proinflammatory cytokines production when compared with the unstimulated condition. The level of cytokines produced was measured by ELISA for TNF-α, IL-6, IL1-β, and IL-8 (Fig. 4). We can observe that the amount of cytokines produced tends to increase with time except for IL6. It is worth noting that the level of IL6 measured in the stimulated conditions is comparable with the unstimulated condition (Fig. 4*A*). This suggests that there was no LPS-induced IL6 production or that the amount produced was too low to be detected by the ELISA test used. The amount of IL1-β also increased with time, but the highest level is reached at 24 h of incubation because a decrease is observed at 48 h of incubation time (Fig. 4*D*). It is also interesting to see that the level of IL8 was particularly high (Fig. 4*C*) when compared with the other cytokines. However, no significant difference was noticed between the parent strain and the mutants because purified LPS from the rough LPS mutant, the core LPS mutants, and the wild type parent strain seem to stimulate equally the production of cytokines by PAMs (Fig. 4). These results also show that the LPS of *E. coli* and the LPS of *A. pleuropneumoniae* serotype 1 exhibited comparable stimulatory activities. Moreover, stimulation of alveolar macrophages with heat-killed bacteria of the wild type parent strain and the LPS mutants gave similar results (data not shown).

RT-PCR data showed an early expression of all the cytokines tested because the level of mRNA reached its peak value at 2 h poststimulation and declined shortly thereafter (Fig. 5). A second peak of lower intensity (IL6, TNF-α, MCP-1, and IL-8) or greater intensity (IL1-β) was also observed at 12 h poststimulation, which is probably because of an autoactivation process caused by the increase of cytokines in the media. It is interesting to mention that this early production of mRNA is in good correlation with the ELISA test because the amount of cytokines pro-

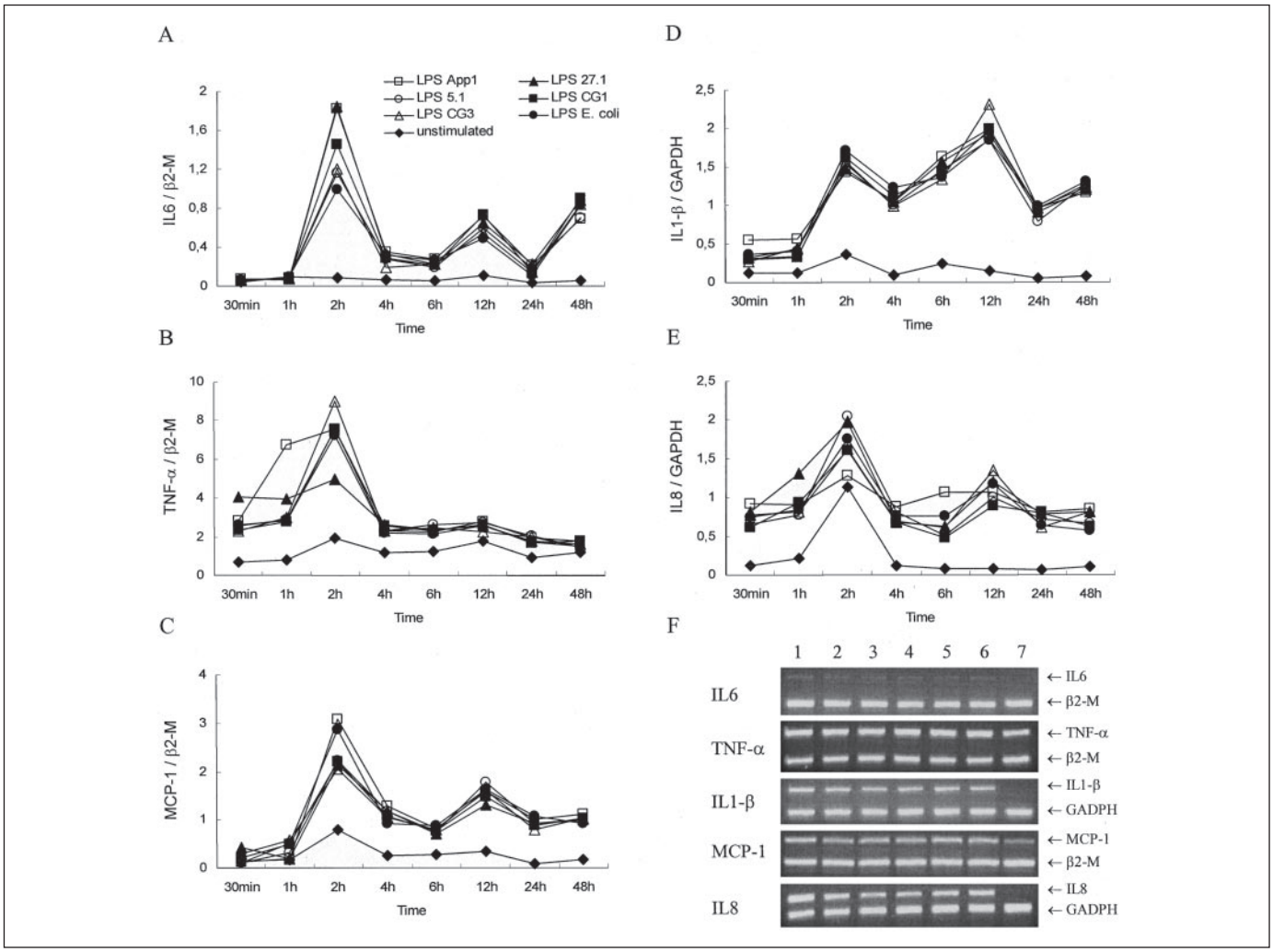

FIGURE 5. Kinetics of IL6 (*A*), TNF-α (*B*), MCP-1 (*C*), IL1-β (*D*), and IL8 (*E*) mRNA expression are shown. Cytokine gene expression levels were determined by RT-PCR in unstimulated and LPS-stimulated PAMs at different times of incubation. Data are represented as a ratio of cytokine/β_2-microglobulin for IL6, TNF-α, and MCP-1 and a ratio of cytokine/GAPDH for IL1-β and IL8. The gels in *F* represent the band intensities of the PCR products migration of RT-PCR analysis for each cytokine after 6 h of incubation. PAMs were stimulated with purified LPS of the parent strain (*lane 1*), the rough LPS mutant 27.1 (*lane 2*), the core LPS mutant 5.1 (*lane 3*), the core LPS mutant CG1 (*lane 4*), the core LPS mutant CG3 (*lane 5*), *E. coli* (*lane 6*), or were unstimulated (*lane 7*).

TABLE FIVE

Minimum inhibitory concentration of various cationic peptides tested with *A. pleuropneumoniae* serotype 1 parent strain, acapsular mutant 33.2, rough LPS mutant 27.1, and core LPS mutants 5.1, CG1, and CG3

Strain	MIC					
	Polymyxin B	Protamine	Mastoparan	Melittin	Cecropin P1	Protegrin-1
			μg/ml			
Parent	0.5–1	250	100	50	5	50
33.2 (CPS$^-$)	0.5	250	50–100	50	5	50
27.1 (rough LPS)	0.5	250	50	25–50	5	50
5.1 (*galU*$^-$)	0.125	62.5	25	12.5	2.5	6.25
CG1 (wlaC$^-$)	0.25	125	50	12.5–25	2.5	12.5
CG3 (lbgB$^-$)	0.25	125	50	12.5	2.5	6.25

duced tends to be higher in the late phase of the stimulation (Fig. 4). Here again, the kinetics of gene expression for each cytokine was identical in all LPS-stimulated conditions (Fig. 5), and these observations confirm the data obtained from ELISAs. However, the quantification of mRNA showed clearly that there is an LPS-induced IL6 expression (Fig. 5*A*) when compared with the unstimulated condition that was undetectable in ELISA. In Fig. 5*F* we can estimate the intensity of the bands, which illustrates the difference between stimulated and unstimulated conditions, and no obvious variations were found among the LPS-stimulated conditions. Once more, the experiment with heat-killed bacteria gave similar RT-PCR results (data not shown). To evaluate further the importance of LPS in the activation of cytokines production, a stimulation experiment was carried out by incubating the PAMs for 24 h with purified LPS in the presence of polymyxin B, an LPS inhibitor. The

TABLE SIX

Results of the experimental infections in pigs

Strain	No. pigs	Mortality[a]	Fever[b]		Average daily gain[c]	Pigs with lung lesions[d]	Average lungs weight[e]	Bacterial isolation[f]		
			% of pigs	Duration				Lungs	Nasal cavities	Tonsils
		%		*days*	*g*	%		%	%	%
Parent	4	75	100	8	−225	100	902.4	100	100	100
Mutant CG1	5	100	100	4	−514	100	1079.0	100	80	100
Mutant CG3	5	20	100	4	−87	100	822.2	100	40	40

[a] Values represent the percentage of dead and sacrificed pigs that had reached a severe stage of the disease within 15 days.
[b] Temperature of >40 °C.
[c] Daily average gain 8 days after infection (in g) is shown.
[d] Percentages are from macroscopic and microscopic examinations.
[e] Averages were compared with ~500 g for noninfected control pigs of the same age.
[f] Data represent the percentage of pigs in which *A. pleuropneumoniae* was isolated from lungs, tonsils, and nasal cavities.

results indicate that the addition of polymyxin B to LPS-stimulated PAMs produced a significant decrease in IL6, TNF-α, MCP-1, and IL1-β gene expression, because the level of mRNA for each of these cytokines tends to drop down to the unstimulated condition values (data not shown). These data confirm that the production of cytokines by PAMs was indeed induced by LPS. Moreover, the decrease of mRNA appeared to be similar in all the LPS-stimulated conditions. This suggests that polymyxin B had the same inhibition effect on purified LPS from the parent strain and the LPS mutants and also on the LPS of *E. coli*. Overall, our data indicate that the LPS of all the mutants tested exhibited the same capacity to stimulate cytokines production as the LPS of the parent strain.

Susceptibility to Antimicrobial Peptides—The susceptibility test was carried out with the three LPS core mutants (5.1, CG1, and CG3), and the following two additional mutants of *A. pleuropneumoniae* serotype 1, previously characterized by our group, were also added to the study: an acapsular mutant 33.2 (34) and a rough LPS mutant 27.1 lacking O-antigen (13, 15). The MIC values obtained (TABLE FIVE) showed that the acapsular mutant (33.2), the rough LPS mutant (27.1), and the wild type parent strain exhibited similar MICs for all the cationic peptides shown in TABLE FIVE, suggesting that the absence of O-antigen of the LPS or the absence of the capsule has no effect on the susceptibility of *A. pleuropneumoniae* serotype 1 to antimicrobial peptides. However, the data also indicate that the three core LPS mutants (5.1, CG1, and CG3) were more susceptible than the parent strain to almost all the peptides tested. Most interestingly, this suggests a role of the outer core in the resistance of *A. pleuropneumoniae* serotype 1 to antimicrobial peptides. It is worth noting that the largest difference of susceptibility between the core LPS mutants and the wild type strain was observed with protegrin-1 (TABLE FIVE), a peptide present in porcine neutrophils.

The experiment was also carried out with a few other peptides that are cited in TABLE THREE. Magainin-1 and indolicidin were tested with the parent strain at 100 μg/ml, but no susceptibility was observed at this concentration. However, it is important to mention that the *E. coli* control strain was susceptible to these two peptides at 50 and 25 μg/ml, respectively, which confirms that the resistance of the *A. pleuropneumoniae* strain was not because of an alteration of the activity of the peptides. Bacitracin was also used and showed a MIC of 250 μg/ml for both the parent strain and the mutants. This is probably because of the site of action of bacitracin that affects mostly the cell wall synthesis. Thus a modification of LPS should not affect the activity of bacitracin.

Experimental Pig Infection—The virulence of the core LPS mutants CG1 and CG3 and the parent strain 4074 Nal^r was evaluated in pigs. After challenge with the three strains, the mortality recorded within the 14 days was high in the mutant CG1-infected group (100%) and the parent strain-infected group (75%), as compared with the mutant CG3-infected group in which only one pig died (20%) (TABLE SIX). Three of the five pigs infected with mutant CG1 died in less than 2 days after infection, whereas only one pig infected with mutant CG3 died and only on day 4 post-infection. As observed in TABLE SIX, the body temperature was higher than normal for all the infected pigs; however, for almost all the pigs that survived, the fever tended to decrease after the 4th day post-infection, except for the parent strain-infected pigs that exhibited a high temperature until 8 days after infection. Dyspnea, anorexia, and in a few cases cough were observed in all infected pigs. Moreover, the performance analysis of the animals was also carried out and showed that pigs infected with the parent strain and the mutant CG1 were more affected than pigs infected with the mutant CG3, in terms of daily average weight gain (TABLE SIX).

Upon macroscopic examination, typical fibrinohemorrhagic lesions compatible with *A. pleuropneumoniae* infection (35) were observed in all infected pigs (TABLE SIX), and the severity of the lesions observed was approximately equal between the three groups. However, the weight of the lungs was also evaluated (TABLE SIX), and the average weight was found to be lower for pigs infected with the core LPS mutant CG3; this could be because of a less intensive inflammation, which would result in a decrease of edema and accumulation of fibrin and blood.

Microscopic examination of histological samples of the lungs revealed changes compatible with porcine pleuropneumonia. Fibrinohemorrhagic pleuresia and pneumonia were associated with leukocytes infiltration and pus formation. The interlobular septum was thickened with edema fluid and inflammatory exudates. Central necrotic zones within the pulmonary tissue were often surrounded by a high density of inflammatory cells, and a certain amount of thrombosed and necrotic lymphatic vessels was also found. It should be noted that the pigs infected with the mutant CG3 showed characteristic lesions of the chronic form of *A. pleuropneumoniae* infection, whereas typical acute phase lesions were observed in mutant CG1-infected pigs that died earlier. *A. pleuropneumoniae* was detected in the lungs of all infected pigs either by PCR or culture (TABLE SIX). Bacterial culture from nasal cavities and tonsils showed that bacteria were not isolated in a large number of pigs infected with the mutant CG3 (TABLE SIX), and interestingly, these pigs survived until the end of the experiment suggesting that the attenuation of the virulence could be because of an insufficient colonization of the host airways by this mutant strain.

Finally, antibodies against *A. pleuropneumoniae* serotype 1 were detected 1 week after infection in the serum of all pigs that survived, and the amount of antibodies tended to increase in the 2nd week (data not

shown). It is worth noting that the growth curves in liquid culture for the parent strain and the two LPS core mutants were identical (data not shown), suggesting that the difference observed in the virulence of these strains was not a matter of growth. The results of this experimental infection study showed clearly that the virulence of mutant CG3 is attenuated when compared with the wild type parent strain or the mutant CG1. In combination with the structural analysis of the core oligosaccharide, the data suggest an important role of the outer core residues Gal I and Hep IV, which are missing in the mutant CG3 but present in mutant CG1 (Fig. 1, *D* and *B*), in the pathogenesis of *A. pleuropneumoniae*.

DISCUSSION

The structural analysis of the core oligosaccharide of the three LPS core mutants has brought additional information on the different LPS biosynthesis genes that were inactivated by the transposon insertion. The analysis of the core of mutant CG1 revealed the loss of the terminal GalNAc-Gal II disaccharide when compared with the parent strain. The core of mutant 5.1 is one hexose more truncated than that of CG1 because of the loss of a galactose residue (Gal I), which also prevents the addition of the terminal disaccharide. Mutant CG3 was found to have the most truncated LPS core as it lacks a D,D-Hep (Hep IV), which is essential for the further addition of the galactose (Gal I and Gal II) and GalNAc residues. These structural analyses correlate with the SDS-PAGE profiles, because the LPS of mutant CG3 has the fastest migration of the core-lipid A region (Fig. 2). The genes affected by the mutations had been identified previously as genes involved in LPS biosynthesis. In the mutant CG1, the gene affected encodes a protein with 25% homology with the WlaC protein of *Campylobacter jejuni*, which is a galactosyltransferase (36). A good correlation was also observed between the sugar deletions within the core structure and the genes affected in the mutants 5.1 and CG3. *galU* (mutant 5.1) is the structural gene for UTP-α-D-glucose-1-phosphate uridylyltransferase, an enzyme involved in the synthesis of UDP-glucose (37), which is also found in *Haemophilus ducreyi* and *Haemophilus influenzae* (87 and 83% identity, respectively), whereas *lbgB* (mutant CG3) encodes a D-glycero-D-mannoheptosyl transferase (38).

The results of the experimental infections reveal that the core LPS mutant CG3 seems to be less virulent than the parent strain with only 20% mortality. Moreover, previous studies by our group have shown that the virulence of the core LPS mutant 5.1 was also attenuated with no mortality recorded, less lung lesions, and also less fever (13). These data are of interest because the two most truncated outer core mutants (5.1 and CG3; Fig. 1, *C* and *D*) are both less virulent than the parent strain; this suggests that the Gal I-Hep IV region of the core plays an important role in the virulence of *A. pleuropneumoniae* serotype 1. Furthermore, previous *in vitro* adhesion experiments using frozen tracheal sections of pigs have demonstrated that the mutants 5.1 and CG3 were both less adherent than the parent strain (13, 14). Thus, it is tempting to speculate that a defect in colonization of the respiratory tract is responsible, at least in part, for the attenuation of virulence.

Previous studies have shown the important role of LPS in stimulating the production of proinflammatory cytokines that were found to be highly produced during *A. pleuropneumoniae* infection (20, 21). It is reasonable to believe that LPS mutants could be altered in their capacity to stimulate cytokines production, and this could have an effect on the pathogenesis of *A. pleuropneumoniae*. The kinetic study of cytokines production has shown that the LPS mutants have the same ability as the wild type parent strain to stimulate the synthesis of cytokines by alveolar macrophages. These findings indicate that mutations within the O-antigen or the core oligosaccharide of LPS have no effect on the ability of *A. pleuropneumoniae* to stimulate cytokines production by immune cells. This is to be expected considering that the endotoxic portion of LPS is the lipid A (39). Because rough LPS and core LPS mutants of *A. pleuropneumoniae* serotype 1 have an identical lipid A, they are not affected in their stimulatory activities. Therefore, truncation of the outer core has no effect on lipid A activity. We can assume that the attenuation of the virulence of both LPS core mutants 5.1 and CG3 is not because of a decreased ability to stimulate production of proinflammatory cytokines. Most interestingly, the measurement of cytokine production showed a high level of IL8 in response to LPS stimulation. This elevated amount of IL8 can be correlated with the massive infiltration of neutrophils in the lungs during *A. pleuropneumoniae* infection (17), because IL8 is the major chemoattractant of these cells (23, 40).

LPS molecules are the major targets of cationic peptides that, most commonly, form disrupting channels in the outer membrane of Gram-negative bacteria and gain access to the plasma membrane (41). Thus, the stability of the outer membrane depends mainly on the good arrangement of the LPS, which is known to involve ionic interactions with bivalent cations (26). Previous studies have shown the important role of Mg^{2+} in tightening the LPS packing within the membrane but also in its capacity to interfere with the binding of the peptides to the negative charges of the LPS (42). This suggests that a mutation in the LPS could alter the integrity of bacterial surface, because of a modification in the ionic interactions, rendering the bacteria more susceptible to antimicrobial peptides. It has also been demonstrated that the core oligosaccharide, rather than the O-antigen, is an important feature in the resistance of *Vibrio cholerae* and *Sinorhizobium meliloti* to such peptides (43, 44), and our findings with *A. pleuropneumoniae* serotype 1 LPS mutants confirm these trends. Our results also showed that the acapsular mutant 33.2 exhibited the same susceptibility as the parent strain to all the peptides tested. This suggests that the capsule is not of major importance in the resistance of *A. pleuropneumoniae* to antimicrobial peptides, although previous studies on *Klebsiella pneumoniae* had shown its protecting role (45). Most interestingly, the core mutants do not always share the same pattern of MIC, depending on the peptides. Because these core mutants are affected in different biosynthesis genes (13, 14), we can assume that the lack of specific sugar residues in the outer core is a determining point in the resistance/susceptibility of *A. pleuropneumoniae* to antimicrobial peptides, as described previously with the regulation of lipo-oligosaccharide structure in *Yersinia pestis* (46). Finally, the *in vitro* test showed that antimicrobial peptides of different species (TABLE THREE) are active against *A. pleuropneumoniae*. The results also revealed that the susceptibility of *A. pleuropneumoniae* to these peptides is not dependent on their structure because α-helical peptides (cecropin P1, melittin, and mastoparan) or peptides with a β-sheet structure (protegrin-1) both inhibited the growth of *A. pleuropneumoniae* serotype 1.

We have characterized the structure of the core oligosaccharide of three core LPS mutants of *A. pleuropneumoniae* serotype 1, previously gene-rated using transposon mutagenesis. The present results confirm that the genes identified are indeed involved in the biosynthesis of the outer core LPS of *A. pleuropneumoniae* serotype 1. The *in vitro* assay using antimicrobial peptides showed the importance of the LPS outer core in the resistance of the bacteria to innate defense components of the host, whereas structural analyses in combination with *in vivo* experiments in the natural host allowed us to determine that some residues of the outer core are particularly important in the pathogenesis of *A. pleuropneumoniae*. To the best of our knowledge, this is the first demon-

stration of the importance of LPS outer core residues in the virulence of a bacterial pathogen for its natural host.

Acknowledgments—We thank Dr. R. I. Lehrer (Dept. of Medicine, UCLA School of Medicine) and Dr. Tony Hayes (Dept. of Pathobiology, University of Guelph, Ontario, Canada) for having provided protegrin-1. We also thank A. Labbé, R. Cariolet, and B. Beaurepaire for their help with the experimental infections and Dr. Jianjun Li for mass spectrometry.

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Truncation of the Lipopolysaccharide Outer Core Affects Susceptibility to Antimicrobial Peptides and Virulence of *Actinobacillus pleuropneumoniae* Serotype 1

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J. Biol. Chem. 2005, 280:39104-39114.
doi: 10.1074/jbc.M502852200 originally published online September 27, 2005
