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Actinobacillus pleuropneumoniae Iron Transport and Urease Activity: Effects on Bacterial Virulence and Host Immune Response

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Actinobacillus pleuropneumoniae*, a porcine respiratory tract pathogen, has been shown to express transferrin-binding proteins and urease during infection. Both activities have been associated with virulence; however, their functional role for infection has not yet been elucidated. We used two isogenic *A. pleuropneumoniae* single mutants (Δ*exbB* and Δ*ureC*) and a newly constructed *A. pleuropneumoniae* double (Δ*ureC* Δ*exbB*) mutant in aerosol infection experiments. Neither the *A. pleuropneumoniae* Δ*exbB* mutant nor the double Δ*ureC* Δ*exbB* mutant was able to colonize sufficiently long to initiate a detectable humoral immune response. These results imply that the ability to utilize transferrin-bound iron is required for multiplication and persistence of *A. pleuropneumoniae* in the porcine respiratory tract. The *A. pleuropneumoniae* Δ*ureC* mutant and the parent strain both caused infections that were indistinguishable from one another in the acute phase of disease; however, 3 weeks postinfection the *A. pleuropneumoniae* Δ*ureC* mutant, in contrast to the parent strain, could not be isolated from healthy lung tissue. In addition, the local immune response—as assessed by fluorescence-activated cell sorter and enzyme-linked immunosorbent spot analyses—revealed a significantly higher number of *A. pleuropneumoniae*-specific B cells in the bronchoalveolar lavage fluid (BALF) of pigs infected with the *A. pleuropneumoniae* Δ*ureC* mutant than in the BALF of those infected with the parent strain. These results imply that *A. pleuropneumoniae* urease activity may cause sufficient impairment of the local immune response to slightly improve the persistence of the urease-positive *A. pleuropneumoniae* parent strain.

Actinobacillus pleuropneumoniae is the etiologic agent of porcine pleuropneumonia, a highly infectious disease of fattening pigs occurring worldwide (12). A number of putative virulence factors, such as Apx toxins, capsule, lipopolysaccharide (LPS), the ability to utilize transferrin-bound iron, and urease, have been described elsewhere (18). To date, conclusive evidence obtained by challenge experiments has been presented to confirm the role of Apx toxins and capsular material. A spontaneous Apx toxin-negative *A. pleuropneumoniae* strain was shown to be avirulent (14), and this result was supported later by using transposon mutagenesis (36) as well as by an isogenic *A. pleuropneumoniae apxC* insertion mutant (29). Also, capsule-deficient *A. pleuropneumoniae* strains obtained by chemical mutagenesis were shown to be attenuated (22), and this result was confirmed by reconstituting virulence properties and capsule formation upon transformation with a recombinant plasmid (39). Also, it was shown recently that the [Cu,Zn]-superoxide dismutase is not required for virulence (35). For other putative virulence factors, such as LPS (1, 3, 4), and the utilization of transferrin-bound iron (15, 17, 40), no conclusive challenge experiments have been performed to date. With respect to urease, data are inconclusive; urease-negative *A. pleuropneumoniae* mutants have been found to produce acute infection (37), whereas in a low dose challenge trial reported recently, a urease-negative mutant was found to be unable to establish infection (7).

The construction of two isogenic *A. pleuropneumoniae* mutants, one that was unable to utilize transferrin-bound iron (Δ*exbB*) (38) and one that was urease negative (Δ*ureC*) (28), was reported previously. For the present communication we have constructed an isogenic double (Δ*ureC* Δ*exbB*) mutant, and we performed an aerosol challenge on *A. pleuropneumoniae*-free pigs using these three mutants and the parent strain. We show a possible role for urease in chronic *A. pleuropneumoniae* infection and demonstrate that utilization of transferrin-bound iron is important for *A. pleuropneumoniae* virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains, plasmids, and primers used in this work are listed in Table 1. *Escherichia coli* strains were cultured in Luria-Bertani medium supplemented with the appropriate antibiotic (ampicillin, 100 μg/ml); for cultivation of *E. coli* β2155 (Δ*dapA*), diaminopimelic acid (1 mM) (Sigma Chemical Company, Deisenhofen, Germany) was added. *A. pleuropneumoniae* serotype 7 parent and mutant strains were grown in PPLO medium (Difco GmbH, Augsburg, Germany) supplemented with NAD (10 μg/ml) (Merck AG, Darmstadt, Germany), L-glutamine (100 μg/ml) (Serva, Heidelberg, Germany), L-cysteine hydrochloride (260 μg/ml) (Sigma), L-cystine dihydrochloride (10 μg/ml) (Sigma), dextrose (1 mg/ml), and Tween 80 (0.1%). Iron restriction was induced by the addition of 2,2′-dipyridyl (100 μM) (Sigma).

Manipulation of DNA and construction of an *A. pleuropneumoniae* double deletion mutant. DNA-modifying enzymes were purchased from New England Biolabs (Bad Schwalbach, Germany) and used according to the manufacturer's instructions. *Taq* polymerase was purchased from GIBCO-BRL Life Technologies (Karlsruhe, Germany). DNA for PCR and Southern blotting, as well as plasmid DNA, was prepared by standard protocols (33). Transformations, gel electrophoresis, PCR, and Southern blotting were done by standard procedures

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TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Characteristics	Reference or source
Strains		
E. coli		
DH5αF′	F′ *endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)*U169 deoR* [ϕ80d*lac*Δ(*lacZ*)*M15*]	30
HB101	F$^-$ Δ(*gpt-proA*)*62 leuB6 supE44 ara-14 galK2 lacY1* Δ(*mcrC-mrr*) *rpsL20* (Str^r) *xyl-5 mtl-1 recA13*	33
β2155	*thrB1004 pro thi strA hsdS lacZ*Δ*M15* (F′ *lacZ*Δ*M15 laqI*^{q} *traD36 proA*$^+$ *proB*$^+$) Δ*dapA::erm* (Erm^r) *recA::RP4-2-tet* (Tc^r)::Mu-km (Km^r) λ *pir*	9
A. pleuropneumoniae		
AP76 (wt)	*A. pleuropneumoniae* serotype 7 strain kindly provided by the Western College of Veterinary Medicine, Saskatoon, Canada	2
A. pleuropneumoniae Δ*exbB*	Unmarked *exbB*-negative knockout mutant of *A. pleuropneumoniae* AP76	38
A. pleuropneumoniae Δ*ureC*	Unmarked *ureC*-negative knockout mutant of *A. pleuropneumoniae* AP76	28
A. pleuropneumoniae Δ*exbB* Δ*ureC*	Unmarked *exbB*- and *ureC*-negative knockout mutant of *A. pleuropneumoniae* AP76	This work
Plasmid		
pBMK1	Transconjugation vector based on pBluescript SK with *mob*RP4, a polycloning site, Km^r, and transcriptional fusion of the *omlA* promoter with the *sacB* gene	28
Primers		
BA7	CAA TGG ATC CAT TTT ATC TTC TTC AGG C; primer (internal *Bam*HI site) upstream of the *exbB* gene	38
RE1	AAG TTT AAA ATG CAT ATT GC; primer overlapping the start codon of the *tbpB* gene	38
ureC2	GTA AGG ATC CAT TAA CAA TCC CAC GCA GTC AGT AT; primer (internal *Bam*HI site) comprising positions 997 to 1022 of the urease operon (reference 6)	28
ureX	TCA TGT CGA CTA GAA CAA GAA ATA ACG CTG TGC AA; primer with internal *Sal*I site comprising positions 2686 to 2711 of the urease operon (reference 6)	28

(33), and pulsed-field gel electrophoresis (PFGE) of *A. pleuropneumoniae* was performed as described previously (27). The *A. pleuropneumoniae* double (Δ*ureC* Δ*exbB*) mutant was constructed using the *A. pleuropneumoniae* urease-negative (Δ*ureC*) mutant (28) as the recipient strain. The plasmids used, as well as conjugation, selection, and counterselection procedures, have been described previously (28, 38).

Virulence studies. Thirty-two outbred pigs 8 to 9 weeks of age were purchased from an *A. pleuropneumoniae*-free herd (no clinical symptoms, no serological response in the ApxII–enzyme-linked immunosorbent assay [ELISA] [24]), randomly assigned to four groups, and cared for in accordance with the principles outlined in the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes* (European Treaty Series, no. 123: http://conventions.coe.int/treaty/EN/Menuprincipal.htm. Groups were housed in separate isolation units with controlled temperature and ventilation. Infections were carried out in an aerosol chamber built according to the descriptions of Jacobsen et al. (23), with four pigs at a time. For aerosol infection, the *A. pleuropneumoniae* parent strain and the isogenic mutants were grown with shaking for approximately 3 h at 37°C to an optical density (OD) at 660 nm of 0.4. The culture was placed on ice, diluted 1:300 in ice-cold NaCl (150 mM), and kept on ice until use (for a maximum of 2 h). Immediately prior to aerosolization, bacteria were further diluted 1:100 in ice-cold NaCl (150 mM), resulting in living cell counts of 9.6×10^4/ml (Δ*ureC* Δ*exbB* mutant), 15×10^4/ml (Δ*exbB* mutant), 9.7×10^4/ml (Δ*ureC* mutant), and 7.4×10^4/ml (wild type [wt]); upon aerosolization these concentrations correspond to approximately 10^2 *A. pleuropneumoniae* cells per liter of aerosol in the chamber, a dose which had been titrated for the *A. pleuropneumoniae* parent strain (wild type) to induce severe but not fatal disease in this challenge model, at a total exposure time of 45 min (2 min of aerosolization, 10 min of incubation, 30 min of removal of bacteria through filters at a rate corresponding to ten complete exchanges of the air volume). Blood samples were taken on day 7 before infection (immediately upon arrival in the facility), as well as on days 7, 14, and 21 postinfection.

At postmortem analysis, lung lesion scores were determined according to the method described by Hannan et al. (19). Briefly, the size and position of lesions were mapped on a diagram representing the seven lung lobes, with each lobe allotted a maximum possible score of 5. Then, by assessing the pneumonic area for each lobe as a fraction of 5 (resulting in a maximum score of 35 for the complete lung), the lung lesion score was calculated. The bacteriological examination included surface swabs of affected and unaffected lung tissue, tonsils, bronchial lymph nodes, and heart muscle on supplemented PPLO agar, as well as on Gassner and Columbia sheep blood (CSB) agar. The degree of total bacterial colonization (growth on CSB agar), as well as colonization by enterobacteria (growth on Gassner agar) and by *A. pleuropneumoniae*-like bacteria (minimal growth with distinct hemolysis on CSB, good growth on supplemented PPLO agar) was assessed as +++ (confluent), ++ (>100 colonies), and + (<100 colonies). Some individual *A. pleuropneumoniae*-like colonies were subcultured on supplemented PPLO agar and confirmed by a slide agglutination test and PCR analysis using *exbB*- and *ureC*-specific primers.

BALF. Pigs were anesthetized by intramuscular application of azaperone (2 mg/kg of body weight) followed by an intramuscular injection of ketamine (15 mg/kg) and immobilized as previously described (21). To obtain bronchoalveolar lavage fluid (BALF), a flexible bronchoscope (type XP20; Olympus, Hamburg, Germany) was introduced into the bronchus of the right posterior cranial lobe. The tip of the bronchoscope was pushed into a wedge position to seal the bronchus. Twenty milliliters of isotonic NaCl (prewarmed to 30°C) was injected and recovered by applying a suction force of 20 to 50 kPa using a specially designed vacuum pump (Endoaspirator; System Endoparts, Georg Paudrach, Hanover, Germany). This washing process was repeated five times, and an average of 90 ml of BALF was obtained. The BALF was kept on ice for up to 2 h until the bacteriological status was assessed. Briefly, 1 ml of BALF was centrifuged (6,000 × *g*, 10 min), and the pellet was resuspended in 60 μl of NaCl (150 mM). Twenty microliters was plated on supplemented PPLO agar, as well as on Gassner and CSB agar, and plates were interpreted as described above. In addition, the total bacterial number as well as the number of *A. pleuropneumoniae* cells was assessed by serial 10-fold dilutions of nonconcentrated BALF and plating on CSB and supplemented PPLO agar.

ELISAs. The generalized humoral immune response of pigs was determined in two different ELISAs. In order to assess antibody levels directed against the ApxIIA toxin, a standardized ELISA based on the recombinant *A. pleuropneumoniae* ApxIIA protein as the solid-phase antigen was employed (24). In order to assess antibody levels directed against outer membrane components, an ELISA based on the detergent extract of an iron-restricted *A. pleuropneumoniae* wild-type (wt) culture (16) as the solid-phase antigen was used. The detergent extract was diluted 1:50 in carbonate buffer (50 mM [pH 9.6]); Polysorb 96-microwell plates (Nunc, Roskilde, Denmark) were coated with 100 μl of diluted extract per well at 4°C for 16 h without subsequent blocking. Plates were washed with PBST (150 mM phosphate-buffered saline [PBS] [pH 7.2] containing 0.05%

Tween 20) before the addition of serum, conjugate, and chromogen. Sera were initially diluted 1:100 and then diluted twofold further in PBST in the plates. An internal positive control (a pool of sera taken at 3 weeks postinfection from pigs infected with *A. pleuropneumoniae* wt cells) and a negative control (a pool of sera taken from pigs prior to infection) were used on each plate. Serum dilutions and goat anti-pig peroxidase conjugate (Dianova, Hamburg, Germany) were each incubated for 1 h at room temperature. The ELISA was developed using 2,2-azino-di-[3-ethylbenzithiazoline sulfonate] (ABTS) (Boehringer, Mannheim, Germany) as a substrate. The test was considered valid when the OD of the negative serum at a 1:100 dilution was lower than the OD of the positive serum at a 1:12,800 dilution. The titer given is the serum dilution with an OD higher than twice the OD of the negative control serum at a 1:100 dilution.

Fluorescence-activated cell sorter and enzyme-linked immunosorbent (ELI) spot analyses. Eighty milliliters of BALF was centrifuged ($400 \times g$, 10 min), and the cells were washed once in PBS and then resuspended in 1.5 ml of PBS. Using phase-contrast microscopy (500-fold magnification) and a hemocytometer, the numbers of lymphocytes, red blood cells, and other nucleated cells (including macrophages and granulocytes) were determined. An indirect immunofluorescence staining method for lymphocyte subpopulations in the BALF cells was performed using monoclonal porcine-specific antibodies against CD3 (8E6; VMRD, Pullman, Wash.), γ/δ T cells (MAC320; R. M. Binns, Babraham, United Kingdom), immunoglobulin A (IgA) (MCA638), IgG1 (MCA635), and IgM (MCA637) (all Igs were from Serotec, Oxford, United Kingdom). Goat anti-mouse isotype-specific phycoerythrin conjugates were used as secondary antibodies (Southern Biotechnologies, Birmingham, Ala.). Using a flow cytometer (FACScan; Becton Dickinson, Heidelberg, Germany) within the lymphocyte gate, the percentage of positive cells for the different markers was determined based on 5,000 analyzed events.

Cells from the BALF were assayed for antibody-secreting cells (ASC) of the different immunoglobulin isotypes (IgA, IgG1, and IgM) and for *A. pleuropneumoniae*-specific ASC of the different isotypes by ELI spot analysis (11). Briefly, nitrocellulose-bottomed 96-well plates (MAHB-N45; Millipore, Eschborn, Germany) were coated with an *A. pleuropneumoniae* antigen preparation (detergent extract of an iron-restricted *A. pleuropneumoniae* wt culture, diluted 1:10) in PBS for 2 h at 37°C. The plates were washed and blocked using RPMI 1640 containing 5% fetal calf serum. After removal of the block, BALF cells were added, and the plates were incubated overnight at 37°C in a moist atmosphere (5% CO_2). The cells were removed by intense rinsing (PBS with 0.05% Tween 20), and monoclonal antibodies against porcine IgA, IgG1, and IgM (see above) were added for 2 h at 37°C. An anti-mouse IgG1 alkaline phosphatase-labeled conjugate (Southern Biotechnologies) was used as the secondary reagent. The color reaction was carried out using alkaline phosphatase buffer (0.1 M Tris, 0.15 M NaCl, 0.05 M $MgCl_2$ [pH 9.5]) containing nitroblue tetrazolium (30 μg/ml) (Sigma) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (16 μg/ml; Sigma). The frequency of ELI spots was counted using a stereomicroscope (30-fold magnification) and expressed as the number of spots per 10^6 lymphocytes. The means and standard deviations for the lymphocyte subsets, as well as for the ELI spots, were calculated; differences with P of <0.05 in the nonparametric Wilcoxon test were considered significant.

RESULTS

Construction of an isogenic *A. pleuropneumoniae* double (Δ*ureC* Δ*exbB*) mutant. An *A. pleuropneumoniae* double (Δ*ureC* Δ*exbB*) mutant was constructed based on an *A. pleuropneumoniae* single (Δ*ureC*) mutant and confirmed by PCR analyses, Southern blotting, PFGE, urease assay, and Western blotting (Fig. 1). Thereby it was shown that the Kan^r *sacB* cassette can be used to successively introduce multiple site-specific mutations into one *A. pleuropneumoniae* parent strain.

Bacterial reisolation kinetics and pathomorphological changes in challenged pigs. BALF was sampled 1 week before as well as 1 and 3 weeks after challenge. Colonization by bacteria other than *A. pleuropneumoniae*, as assessed by the total count of CFU, was highly variable among individual pigs (<10/ml to 5×10^5/ml), but no significant differences were found among the four challenge groups or between the different sampling points. Upon challenge with the *A. pleuropneumoniae* Δ*exbB* and Δ*ureC* Δ*exbB* mutants, no *A. pleuropneumoniae* cells could be reisolated from BALF 1 or 3 weeks after challenge. Upon challenge with the *A. pleuropneumoniae* Δ*ureC* mutant and the wt strain, the challenge strain was reisolated from BALF on days 7 and 21 from the majority of pigs in these challenge groups (Table 2). No consistent difference with respect to the number of *A. pleuropneumoniae* colonies was observed between the two groups or between days 7 and 21. The correct pheno- and genotype of isolates were confirmed by urease testing and PCR analysis.

The bacteriological examination of tonsils, lung lymph nodes, hearts, pneumonic lesions (if present), and intact lungs at the end of the challenge experiment (3 weeks after challenge) revealed that all pigs challenged with the *A. pleuropneumoniae* Δ*exbB* or Δ*ureC* Δ*exbB* mutant were culture negative (Table 2). From pigs challenged with the *A. pleuropneumoniae* wt and Δ*ureC* strains, *A. pleuropneumoniae* was consistently reisolated from pneumonic lesions in pure culture, with surface smears showing dense (++) or confluent (+++) growth in 13 of 16 pigs. Reisolation from the hearts and tonsils succeeded sporadically, with no differences between the groups. The lymph nodes were culture positive for the wt strain and the Δ*ureC* mutant for four and five pigs, respectively. The morphologically intact lung tissue was culture positive for four pigs challenged with the wt strain, whereas it was culture negative for all pigs challenged with the Δ*ureC* mutant (Table 2).

Systemic and local immune response of challenged pigs. The systemic immune response was determined with two ELISA systems, using recombinant ApxIIA protein or detergent extract as the solid-phase antigen. Among the pigs challenged with the *A. pleuropneumoniae* Δ*exbB* or Δ*ureC* Δ*exbB* mutant, none developed a detectable immune response (Fig. 2). In contrast, all pigs challenged with the *A. pleuropneumoniae* Δ*ureC* mutant or the wt strain showed a strong humoral immune response in both systems, and no significant difference was observed between the groups (Fig. 2). The local immune response was studied based on the cells recovered from BALF. The total BALF volume (approximately 90 ml) contained $\sim 20 \times 10^6$ nonlymphoid cells (mainly macrophages and granulocytes) and $\sim 4 \times 10^6$ lymphocytes for all groups. These cell numbers remained at a comparable level throughout the entire challenge study. Using fluorescence-activated cell sorter analysis, lymphocytes were differentiated before challenge, as well as 1 and 3 weeks after challenge, into T cells ($CD3^+$) and IgM-, IgA-, and IgG-expressing ASC, with no obvious differences among the groups. Using the ELI spot assay with an *A. pleuropneumoniae* detergent extract as the solid-phase antigen, *A. pleuropneumoniae*-specific ASC were detected 3 weeks after challenge. The number of specific ASC was clearly increased in pigs challenged with the *A. pleuropneumoniae* Δ*ureC* mutant compared to all other groups; this increase was significant ($P < 0.05$) for IgM- and IgG-secreting cells (IgM, 10 to 210 ASC/10^6 cells; IgG, 40 to 1,000 ASC/10^6 cells) (Fig. 3).

DISCUSSION

The goal of this study was to determine the roles of putative *A. pleuropneumoniae* virulence factors in persistence and chronic infection. For our approach of using isogenic mutants, two target genes, *exbB* and *ureC*, were chosen. The *exbB* gene was selected because it has been shown previously that dele-

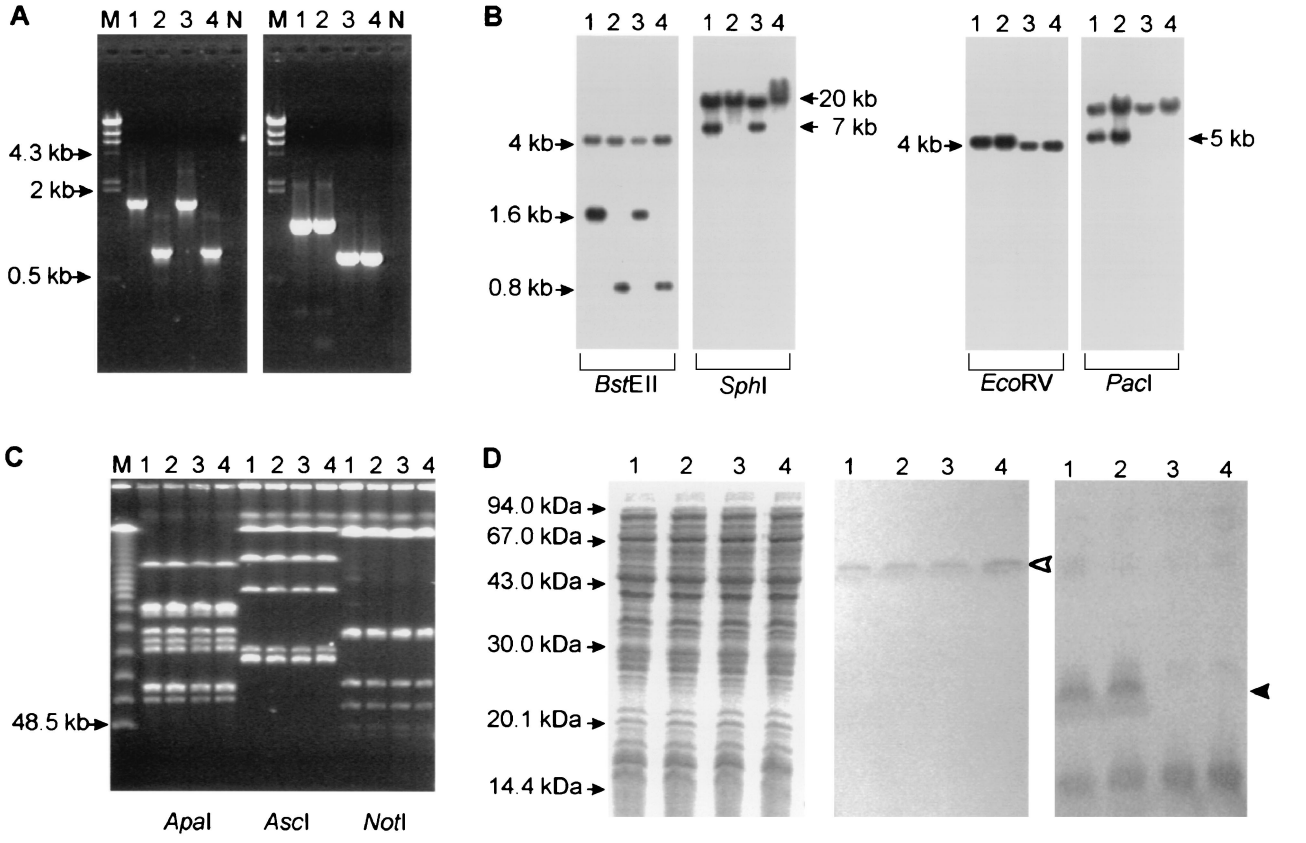

FIG. 1. Analysis of *A. pleuropneumoniae* AP76 wt (lanes 1), Δ*ureC* (lanes 2), Δ*exbB* (lanes 3), and Δ*ureC* Δ*exbB* (lanes 4) strains. Lanes M, size markers; lanes N, negative controls for PCR. (A) PCR using primers ureC2 and ureX (left) and RE1 and BA7 (right). (B) Southern blot analysis using the *ureC* gene (left) and the *exbB* gene (right) as probes. The *ureC* probe is cut by *Bst*EII and *Sph*I, with *Bst*EII located outside and *Sph*I located within the deletion site. The *exbB* probe is not cut by *Eco*RV and *Pac*I, with *Eco*RV located outside and *Pac*I located within the deletion site. (C) PFGE of *Apa*I-, *Asc*I-, and *Not*I-digested DNA showing that no gross rearrangements have occurred. (D) Coomassie blue-stained gel (left) and Western blots developed with serum directed against the TbpB protein (middle) and the ExbB protein (right) of whole cell lysates obtained from cultures grown under iron-restricted conditions, showing that TbpB expression in the *exbB* mutants (Δ*exbB* and Δ*ureC* Δ*exbB*) is unaffected. The open arrowhead indicates the position of the TbpB protein; the solid arrowhead indicates the position of the ExbB protein.

tion of this gene completely inhibits utilization of transferrin-bound iron without preventing the expression of the TbpB protein (38), which is known to be a protective antigen (32). Since the expression of TbpB protein is particularly high in acute infection and decreases during the course of disease (20) and since it has been shown that *A. pleuropneumoniae* can obtain iron from siderophores of other bacteria (10) and, in addition, is able to bind hemin and hemoglobin (3), we hypothesized that the isogenic *exbB* mutant (Δ*exbB*) would be attenuated in the acute phase of disease but would still be able to persist.

The *ureC* gene was selected because acute *A. pleuropneumoniae* infection can occur upon experimental infection using a high challenge dose of a urease-negative *A. pleuropneumoniae* mutant (7, 37), and a urease-negative field strain has been recovered in one case of natural infection (5). For other pathogens causing respiratory tract infections, either no effect (in the case of *Bordetella bronchiseptica* [25]) or a slight decrease in

TABLE 2. Virulence of *A. pleuropneumoniae* parent and isogenic mutant strains following aerosol challenge

A. pleuropneumoniae challenge strain	No. of animals with lung lesions/total no.	Arithmetic mean ± standard deviation of lung lesion score[a]	No. of animals with *A. pleuropneumoniae* reisolated after challenge from:						
			BALF		Tonsil[b,c]	Lymph node[b]	Heart[b]	Lung[b]	
			Day 7	Day 21				Pneumonic	Intact
Δ*ureC* Δ*exbB* mutant	0/8	0	0	0	0	0	0	0	0
Δ*exbB* mutant	0/8	0	0	0	0	0	0	0	0
Δ*ureC* mutant	7/8	5.66 ± 4.02	6	5	1	5	2	7	0
wt	8/8	6.64 ± 2.95	4	7	1	4	0	8	4

[a] The lung lesion score was determined as described by Hannan et al. (19).
[b] Tissues were examined at postmortem analysis.
[c] In tonsil cultures, the presence of *A. pleuropneumoniae* was likely to be masked frequently by the heavy load of concomitant bacterial growth.

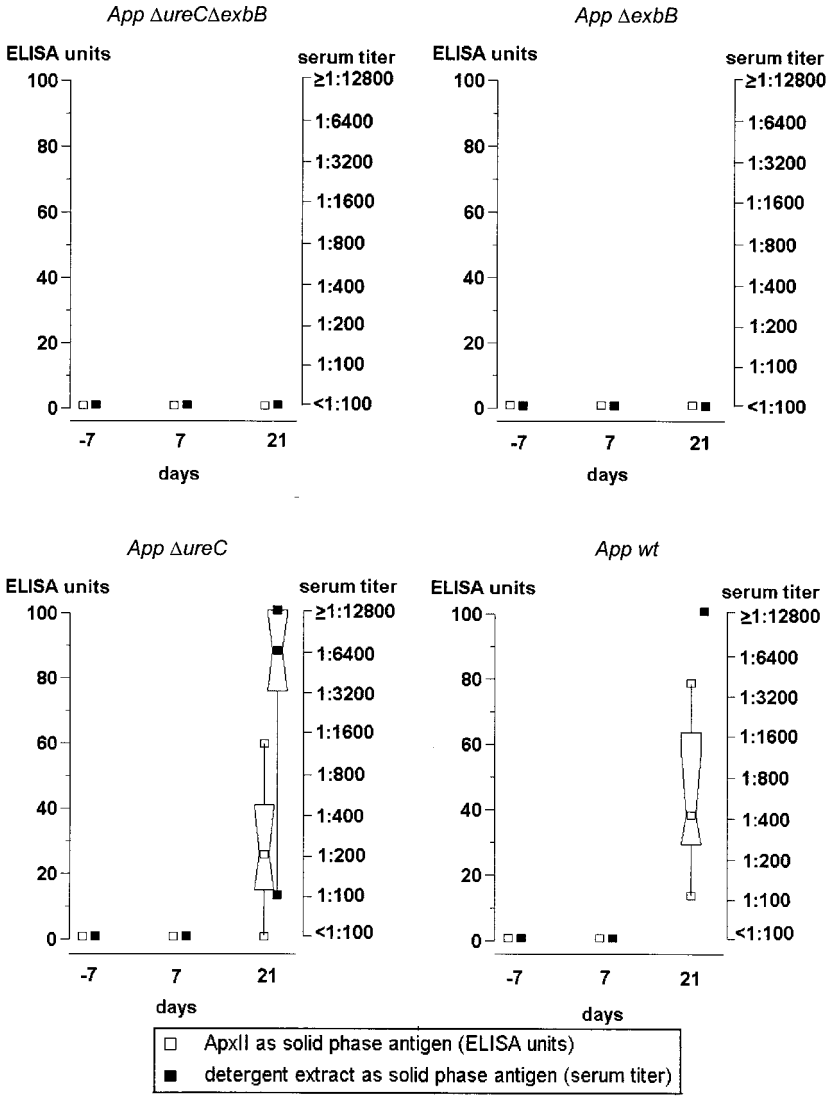

FIG. 2. Humoral immune response of pigs challenged with the *A. pleuropneumoniae* (*App*) parent strain and isogenic mutants 7 days before as well as 7 and 21 days after challenge. The antibody response was assessed with two ELISAs, using the recombinant ApxIIA protein (Apx-ELISA) or a detergent extract (Extract-ELISA) as the solid-phase antigen. The immune response was expressed in ELISA units (based on an external standard) for the standardized Apx-ELISA, with serum activities of more than 30 ELISA units considered positive; for the Extract-ELISA, the immune response was expressed as serum titer in comparison to an internal control. The central square within the hourglass shape represents the geometric mean, the hinges present the values in the middle of each half of data, and the top and bottom squares mark the maximum and minimum values, respectively.

multiplication and persistence (in the case of *Mycobacterium bovis* [31]) has been observed. We hypothesized that urease might support the persistence and shedding of *A. pleuropneumoniae* by locally counteracting the reactive decrease of pH occurring upon infection. Since the RTX toxin of *E. coli* is known to be inhibited by subneutral pH values (34), a local urease-mediated return to physiological pH might maintain or restore the toxic efficacy of the *A. pleuropneumoniae* Apx toxin and thereby impair local defense mechanisms of the host, particularly in the late stage of infection. Based on these considerations, we also constructed the *A. pleuropneumoniae* double (Δ*ureC* Δ*exbB*) mutant which, according to our hypotheses, would be attenuated over the entire course of disease.

The results we obtained with the *A. pleuropneumoniae* Δ*exbB* mutant contradicted our prediction. The complete absence of these mutants in BALF after only 1 week after infection and the lack of any specific humoral or local immune response implies that the ExbBD-mediated uptake of transferrin-bound iron is required for *A. pleuropneumoniae* virulence. The iron uptake via exogenous siderophores (10) is not sufficient to facilitate colonization of the respiratory tract by *A. pleuropneumoniae* or, alternatively, also depends on the ExbBD transporter function. This likely dependence on transferrin-bound iron is supported by results with *Neisseria gonorrhoeae* showing that transferrin receptor mutants were unable to cause infection in human volunteers (8).

The results obtained in the challenge experiment with the *A. pleuropneumoniae* Δ*ureC* mutant confirmed previous results

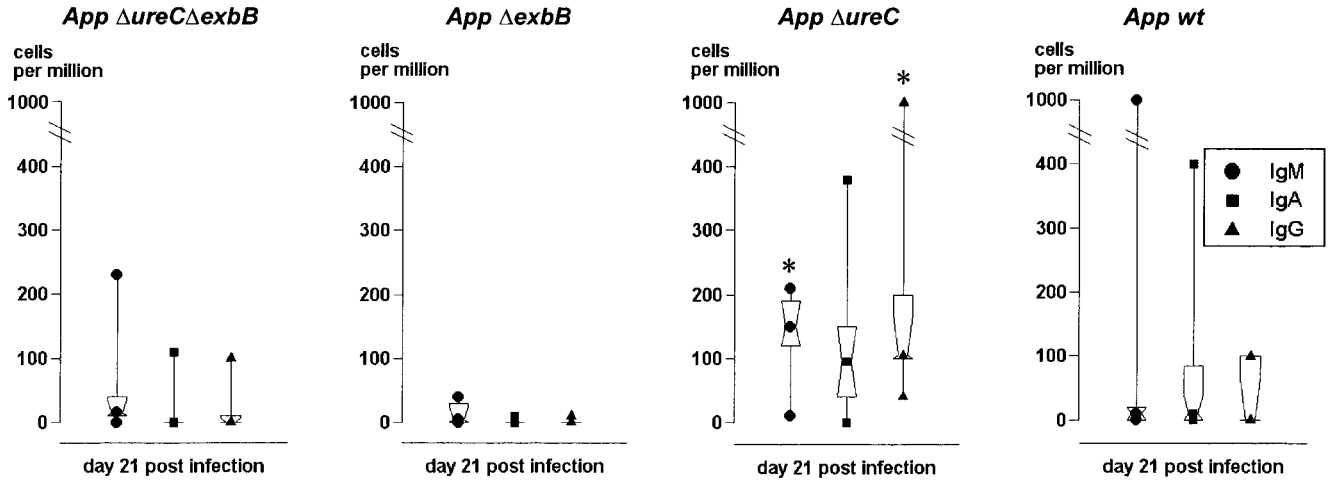

FIG. 3. Local immune response of pigs challenged with the *A. pleuropneumoniae* (*App*) parent strain and isogenic mutants 21 days after challenge. The immune response was assessed by ELI spot analysis differentiating between IgM (●), IgA (■), and IgG (▲) ASC. Significant differences are indicated by an asterisk. The dot, square, or triangle within the hourglass shape represents the geometric mean, the hinges present the values in the middle of each half of data, and the top and bottom symbols mark the maximum and minimum values, respectively.

with respect to acute infection (7, 37) and supported the hypothetical role of urease in chronic infection. The *A. pleuropneumoniae* Δ*ureC* mutant could not be isolated from unaltered lung tissue 3 weeks after challenge (Table 2), and this finding was further supported by the ELI spot assay showing a significantly higher number of *A. pleuropneumoniae*-specific B cells in the BALF from Δ*ureC*-infected pigs than in the BALF from pigs infected with the parent strain (Fig. 3). This difference could be due to a more effective antigen uptake and presentation by dendritic cells in the airways (26), thereby leading to an increased number of ASC; this possibility is supported by the urease function hypothesized above. The similar numbers of total B and T cells do not contradict this potential explanation, as the lytic activity of Apx toxin is concentration dependent and would therefore be expected to primarily affect *A. pleuropneumoniae*-specific cells. To substantiate this hypothesis, however, additional challenge trials using different challenge doses should be performed.

A major obstacle in preventing *A. pleuropneumoniae* disease is the serovar-specific protection induced upon immunization with bacterins. One way to successfully overcome this problem is the use of attenuated live vaccines (13, 29). However, licensing of isogenic mutants containing an antibiotic resistance marker for use in livestock might be difficult to obtain. Therefore, the feasibility of successively introducing multiple mutations without antibiotic markers into the same parent strain might prove valuable for future *A. pleuropneumoniae* vaccine development. Based on our results, a urease-negative phenotype introduced as one mutation might be advantageous, as it facilitates a simple differentiation from common *A. pleuropneumoniae* isolates and, in addition, might reduce shedding of such a putative live vaccine.

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