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Deletion of the Anaerobic Regulator HlyX Causes Reduced Colonization and Persistence of *Actinobacillus pleuropneumoniae* in the Porcine Respiratory Tract

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***Actinobacillus pleuropneumoniae*, the etiological agent of porcine pleuropneumonia, is able to persist on respiratory epithelia, in tonsils, and in the anaerobic environment of encapsulated lung sequesters. We have demonstrated previously that putative HlyX-regulated genes, coding for dimethyl sulfoxide (DMSO) reductase and aspartate ammonia lyase, are upregulated during infection and that deletions in these genes result in attenuation of the organism. The study presented here investigates the role of HlyX, the fumarate nitrate reductase regulator (FNR) homologue of *A. pleuropneumoniae*. By constructing an isogenic *A. pleuropneumoniae hlyX* mutant, the HlyX protein is shown to be responsible for upregulated expression of both DMSO reductase and aspartate ammonia lyase (AspA) under anaerobic conditions. In a challenge experiment the *A. pleuropneumoniae hlyX* mutant is shown to be highly attenuated, unable to persist in healthy lung epithelium and tonsils, and impaired in survival inside sequestered lung tissue. Further, using an *A. pleuropneumoniae* strain carrying the *luxAB* genes as transcriptional fusion to *aspA* on the chromosome, the airway antioxidant glutathione was identified as one factor potentially responsible for inducing HlyX-dependent gene expression of *A. pleuropneumoniae* in epithelial lining fluid.**

Actinobacillus pleuropneumoniae, the etiological agent of porcine pleuropneumonia (10), is able to persist in host tissues, such as tonsillar crypts and sequestered necrotic lung, where the oxygen supply is scarce. The resulting carrier animals are the major source of infection for previously *A. pleuropneumoniae*-free herds (10) and, therefore, unraveling the mechanisms of persistence is highly relevant to effective vaccination and control of the infection.

In *Escherichia coli* a number of genes expressed under anaerobic conditions are regulated by the global regulator FNR (for fumarate nitrate reductase regulator) (24). An *A. pleuropneumoniae* FNR homologue, HlyX, has been found to induce hemolytic activity in *E. coli* under anoxic conditions and to be able to complement *E. coli fnr* deletions (18, 26). Like FNR, HlyX contains four iron-sulfur clusters responsible for the DNA-binding ability of the protein (12).

In *A. pleuropneumoniae*, anaerobically regulated genes appear to play a role in virulence and persistence. We have shown previously that *A. pleuropneumoniae* genes upregulated under anaerobic conditions in culture are not only upregulated in sequestered necrotic lung tissue where anoxic conditions are to be expected (1) but also upon the supplementation of culture medium with bronchoalveolar lavage fluid from *A. pleuropneumoniae*-infected pigs, which mimics conditions as they occur on respiratory epithelium (1–3, 15). Further, we have shown that isogenic mutants lacking aspartate ammonia lyase (aspartase) activity or both dimethyl sulfoxide (DMSO) reductase and aspartase activity were reduced in virulence and in their ability to persist on unaltered respiratory epithelium (2, 15).

Since both the DMSO reductase (*dmsA*) and the aspartase (*aspA*) genes contain putative HlyX-binding motifs (2, 15), we constructed an isogenic *A. pleuropneumoniae* mutant strain lacking the *hlyX* gene, examined it in vitro with respect to the regulation of DMSO reductase expression and aspartase activity, and used the strain in an aerosol infection model in order to examine the effect of the *hlyX* deletion on *A. pleuropneumoniae* virulence and persistence. Further, in order to investigate how genes controlled by the anaerobic regulator HlyX could be upregulated in the aerobic environment of the respiratory epithelium, we examined the influence of a common airway antioxidant, reduced glutathione (GSH), on aspartase expression in a luciferase reporter assay with an *A. pleuropneumoniae* strain carrying the *luxAB* genes in transcriptional fusion to the *aspA* gene on the chromosome (15).

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. The strains, plasmids, and primers used in the present study are listed in Table 1.

Media and growth conditions. *E. coli* strains were cultured in LB medium supplemented with the appropriate antibiotics (ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml); for cultivation of *E. coli* β2155 (Δ*dap*), diaminopimelic acid (1 mM; Sigma-Aldrich, Munich, Germany) was added. *A. pleuropneumoniae* strains were cultured in PPLO medium (Difco GmbH, Augsburg, Germany) supplemented with nicotinamide dinucleotide (10 μg/ml; Merck, Darmstadt, Germany), L-glutamine (100 μg/ml; Serva, Heidelberg, Germany), L-cysteine-hydrochloride (260 μg/ml; Sigma-Aldrich), L-cystine-dihydrochloride (10 μg/ml; Sigma-Aldrich), dextrose (1 mg/ml), and Tween 80 (0.1%) on Columbia Sheep

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

Strain, plasmid, or primers	Characteristics[a]	Source and/or references
Strains		
E. coli β2155	*thrB1004 pro thi strA hsdS lacZ*ΔM15 (F′ *lacZΔM15 laqI*^{q} *traD36 proA*$^+$ *proB*$^+$) Δ*dapA*::*erm* (Erm^r)*recA*::*RPA-2-tet* (Tc^r)::Mu-km (Km^r) λ*pir*	9
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]	21
A. pleuropneumoniae AP76	*A. pleuropneumoniae* serotype 7, field strain	19
A. pleuropneumoniae Δ*hlyX*	Unmarked *hlyX*-negative knockout mutant of *A. pleuropneumoniae* AP76	This study
A. pleuropneumoniae Δ*aspA*::*lux*AB	Unmarked *A. pleuropneumoniae* mutant carrying *luxAB* as a transcriptional fusion in the *aspA* gene	15
Plasmids		
pCR2.1-TOPO	Topoisomerase I enhanced *E. coli* cloning vector	TOPO TA Cloning, Invitrogen, Groningen, The Netherlands (25)
pBluescript SK(+)	3.0-kb cloning vector; Ap^r	Stratagene, La Jolla, CA
pEMOC2	Transconjugation vector based on pBluescript SK with *mob*RP4, polycloning site, Cm^r, and transcriptional fusion of the *omlA* promoter with the *sacB* gene	Accession no. AJ868288 (5)
pHLYX810	2,338-bp PCR product of primers oHLYX5 and oHLYX6, containing the *hlyX* ORF, cloned into pCR 2.1-TOPO	This study
pHLYX110	ApaI/NotI fragment carrying the *hlyX* ORF from pHLYX810, ligated into pBluescript SK(+)	This study
pHLYX111	Deletion of an 883-bp fragment between the BgIII and XcmI restriction sites of pHLYX110	This study
pHLYX701	ApaI/NotI fragment from pHLYX111, ligated into pEMOC2	This study
pLS88	Broad-host-range shuttle vector from *Haemophilus ducreyi*; Str^r Sm^r Km^r	33
pHLYX1300	PCR product generated from primers oHLYX9 and oHLYX10 containing *hlyX* ORF, ligated into plasmid pLS88 after MfeI restriction in a 5′–3′ orientation with respect to the vector-derived *sulII* promoter	This study
pHLYX1301	PCR product generated from primers oHLYX9 and oHLYX10 containing *hlyX* ORF ligated into plasmid pLS88 after MfeI restriction in a 3′–5′ orientation with respect to the vector-derived *sulII* promoter	This study
Primers		
oHLYX5	5′-TGG GGC CCT CGG TAC AAC GGT ATG TCC TT-3′; forward primer containing an ApaI restriction site, situated 986 bp upstream of the *hlyX* start codon	This study
oHLYX6	5′-TCG CGG CCG CCA ACG TGA GAG CTT CGT TCA-3′; reverse primer containing a NotI restriction site, situated 626 bp downstream of the *hlyX* ORF	This study
oHLYX7	5′-TCC GAA ACC GGA TAA TTC AC-3′; forward primer situated 507 bp upstream of the *hlyX* start codon	This study
oHLYX8	5′-AGC GAA AGG GTT AAT CAG CA-3′; reverse primer situated 228 bp downstream of the *hlyX* ORF	This study
oHLYX9	5′-ATG ACA ATT GTT TTA AAA GAC GGT AGC CCT TAT G-3′; forward primer containing an MfeI restriction site, situated 20 bp upstream of the *hlyX* start codon	This study
oHLYX10	5′-CTT ACA ATT GCG CCT ATA CGG TCA GTT CGT-3′; reverse primer containing an MfeI restriction site situated 4 bp downstream of the *hlyX* ORF	This study

[a] Erm^r, erythromycin resistance; Tc^r, tetracycline resistance; Km^r, kanamycin resistance; Str^r, streptomycin resistance; Sm^r, sulfonamide resistance; Km^r, kanamycin resistance.

Blood agar (Oxoid GmbH. Wesel, Germany) or on selective meat-blood agar (16). *A. pleuropneumoniae* transconjugants (single crossovers) and transformants were grown in PPLO medium containing chloramphenicol (5 μg/ml), and the medium for counterselection was prepared as described previously (28).

Anaerobic cultures used for the determination of aspartase activity and DmsA expression were prepared as described previously (2, 15). To compare growth of the different strains under anaerobic conditions, 100 ml of supplemented PPLO medium was preincubated in an anaerobic chamber for at least 48 h, inoculated with a single colony, and further incubated at 37°C for 16 h. Since all *A. pleuropneumoniae* strains used in the present study showed severe clumping under anaerobic conditions, bacterial growth was determined as dry pellet weight in triplicate. Bacteria were harvested by centrifugation, and the pellets were dried at 80°C for 24 h and then weighed. Statistical analysis of pellet weights was performed by using Student's *t* test.

Manipulation of DNA. 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-BR Life Technologies (Karlsruhe, Germany). Chromosomal DNA for PCR and Southern blotting, as well as plasmid DNA, was prepared by standard protocols (22). PCR, Southern blotting, transformation, and gel electrophoresis were done by standard procedures (22), and pulsed-field gel electrophoresis was performed as described previously (19).

Electrotransformation. Electrotransformation was performed by using a GenePulser (Bio-Rad, Munich, Germany) according to a published protocol (30) adapted to *A. pleuropneumoniae*. In brief, 250 ml of supplemented PPLO medium was inoculated with a 1/10 volume of an overnight culture, followed by incubation with shaking to an optical density at 600 nm (OD_{600}) of 0.3. The culture was chilled on ice for 15 min, and bacteria were harvested by centrifu-

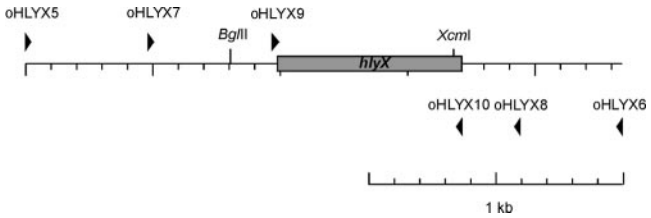

FIG. 1. Map of the *A. pleuropneumoniae* Δ*hlyX* locus with primers and restriction sites used in the present study. Primer binding sites are indicated by arrowheads; restriction sites used are indicated by upward vertical lines.

TABLE 2. Aspartase assay

A. pleuropneumoniae strain	Mean increase in aspartase activity under anaerobic conditions ± SD[a]
Wild type	191 ± 34
Mutant Δ*hlyX*	106 ± 21
Mutant Δ*hlyX* + pHLYX 1300	202 ± 25
Mutant Δ*hlyX* + pHLYX 1301	104 ± 13

[a] Arithmetic mean of results from three independent experiments. The relative activity under aerobic conditions is 100%.

gation at 4,000 × *g* and 4°C. Bacteria were washed three times by centrifugation in 150 ml of ice-cold GYTT medium (10% glycerol, 0.125% Bacto yeast [Difco], 0.25% Bacto tryptone [Difco], 0.02% Tween 80). Finally, bacteria were resuspended in a final volume of 2.5 ml of GYTT medium. Then, 5 μg of salt-free plasmid DNA was added to a 0.4-ml aliquot of competent cells, and electrotransformation was performed in 0.2-cm cuvettes with the settings 2.5 kV, 25 μFa, and 800 W. After electropulsing, bacteria were immediately added to 1 ml of prewarmed, supplemented PPLO medium, followed by incubation for 4 h at 37°C in a 5% CO_2 incubator. Transformants were then plated on PPLO agar with chloramphenicol and incubated for 24 h.

Cloning of the *A. pleuropneumoniae hlyX* gene and construction of an isogenic deletion mutant. The *hlyX* gene (GenBank accession no. M80712) of *A. pleuropneumoniae* was amplified on a 2,338-bp fragment from genomic *A. pleuropneumoniae* AP76 DNA by using primers oHLYX5 and oHLYX6 (Table 1 and Fig. 1), which contained ApaI or NotI restriction endonuclease sites, respectively. After restriction with enzymes ApaI and NotI, the PCR product was ligated into pBluescript SK(+) to obtain plasmid pHLYX100. An 883-bp fragment was then deleted from the *hlyX* gene by using enzymes BglII and XcmI, and the plasmid was religated after fill-in with Klenow fragment to yield plasmid pHLYX101. The truncated *hlyX* gene was removed from pHLYX101 on an ApaI-NotI fragment and ligated into transconjugation plasmid pEMOC2, resulting in plasmid pHLYX701, which was then used to introduce the *hlyX* deletion into *A. pleuropneumoniae* via the single-step transconjugation system as described previously (5, 20) resulting in *A. pleuropneumoniae* Δ*hlyX*.

Complementation of *A. pleuropneumoniae* Δ*hlyX*. Plasmids pHLYX1300 and pHLYX1301 were constructed by cloning the 746-bp MfeI-restricted PCR product generated by primers oHLYX9 and oHLYX10 (Table 1 and Fig. 1), containing the entire *hlyX* open reading frame (ORF) without the putative promoter region, into EcoRI-restricted plasmid pLS88 (33) and electroporated into *A. pleuropneumoniae* Δ*hlyX*. In plasmid pHLYX1300 the orientation of the *hlyX* gene is in the orientation of transcription initiated by the vector-derived *sulII* promoter; in pHLYX1301 it is located in the opposite orientation.

Determination of aspartase and urease activity of *A. pleuropneumoniae*. The aspartase test was performed by determination of fumarate formation as described previously (15). For testing urease activity individual colonies grown on supplemented PPLO agar were overlaid with 0.5% agarose containing 0.3 M urea and 0.01% phenol red (Sigma). The color of the colonies was assessed after 1 min. Urease-positive colonies turned red, whereas urease-negative colonies turned yellow.

Western blot analysis. *A. pleuropneumoniae* whole-cell lysates were analyzed by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10.8% acrylamide and 0.29% bisacrylamide) and Western blotting by using a Protean II Minigel system (Bio-Rad) as described previously (11). The serum directed against DmsA had been raised in rabbits as described previously (2).

Virulence studies. Virulence of *A. pleuropneumoniae* Δ*hlyX* was assessed in an aerosol infection model previously described (4); *A. pleuropneumoniae* free and clinically healthy pigs (German Landrace) 7 to 9 weeks of age were randomly assigned to the different 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; permit no. 009i-(neu)42502-98/45] Clinical examinations were performed daily or as needed. Body temperature and clinical symptoms were recorded daily for each individual pig. A clinical scoring system based on the directive in the European Pharmacopoeia for testing *A. pleuropneumoniae* vaccines (porcine actinobacillosis vaccine [inactivated]) was used to assess the clinical condition of each individual animal as follows: a score of 1 was given for each occurrence of coughing, dyspnea, and vomitus, resulting in a minimum clinical score of 0 and a maximum score of 3 per day; the added daily clinical scores of days 1 to 7 were designated as the total clinical score. Statistical analysis of the total clinical score was performed by using Student's *t* test. In order to confirm the absence of *A. pleuropneumoniae*-specific antibodies, blood samples were taken 1 week prior to infection; blood samples on days 7 and 21 postinfection were taken to determine the serological response to challenge with the different *A. pleuropneumoniae* strains by using the ApxII-ELISA (17). Postmortem analysis, as well as bacteriological, serological, and histological examinations, was performed as described previously (4). Briefly, lung lesion scores were determined as described by Hannan et al. (14) and statistically analyzed by using the Mann-Whitney test. The bacteriological examination included surface swabs of affected and unaffected lung tissue, palatine tonsils, bronchial lymph nodes, and heart muscle on Columbia sheep blood agar and selective meat-blood agar (16). Several individual *A. pleuropneumoniae*-like colonies were subcultured on supplemented PPLO agar and confirmed by urease assay and PCR analysis with primers oHLYX7 and oHLYX8.

Quantitative analysis of *A. pleuropneumoniae* in tonsils and sequestered lung tissue. To determine the number of CFU of *A. pleuropneumoniae* still present 3 weeks after infection, 100 mg of tissue was processed in a FastPrep FP120 instrument (QBiogene, Heidelberg, Germany) with six sterile 3-mm glass beads (Roth, Karlsruhe, Germany) in 1 ml of NaCl (150 mM) twice for 40 s each time at a setting of 5.5. The number of *A. pleuropneumoniae* CFU was assessed by serial 10-fold dilutions and plating on selective meat-blood agar.

Luciferase assay. The *A. pleuropneumoniae* Δ*aspA*::*luxAB* mutant, carrying a transcriptional fusion of *aspA* and *luxAB*, was used in a luciferase assay as described previously (15). To investigate the effect of reduced GSH on transcription of the *aspA* gene, *A. pleuropneumoniae* Δ*aspA*::*luxAB* was grown in liquid culture with shaking at 200 rpm to an OD_{600} of 0.5 to 0.6. This culture was split into 5-ml aliquots, and an equal volume of NaCl (150 mM) containing 1.2 μM, 12 μM, 120 μM, 600 μM, 1200 μM, or 12 mM GSH, respectively, was added to the culture medium. To one aliquot, NaCl without GSH was added to serve as a negative control. The cultures were further incubated with shaking for 1 h, reaching an OD_{600} of ca. 0.7 to 0.8. For the luciferase assay, 2.5 ml of each culture was immediately transferred to a Lumox 24-well plate (In Vitro Systems & Services GmbH, Göttingen, Germany). After the addition of 5 μl of 1% *N*-decyl-aldehyde (Sigma-Aldrich), the plate was exposed to X-ray film for 3 min. Signals were quantified by using Multi-Analyst/PC software (Bio-Rad).

RESULTS

Construction and functional characterization of the isogenic mutant *A. pleuropneumoniae* Δ*hlyX* in vitro. A 883-bp deletion eliminating the 5′ end of the *hlyX* gene (Fig. 1) was introduced into *A. pleuropneumoniae* AP76 via conjugation with plasmid pHLYX701, followed by sucrose counterselection as described previously (28). The resulting *A. pleuropneumoniae hlyX* mutant was verified by using PCR, Southern blotting, and pulsed-field gel electrophoresis analyses (data not shown).

Due to the tendency of *A. pleuropneumoniae* to form clumps under anaerobic conditions, the difference in growth rate was assessed by comparison of dry pellet weights. Under anaerobic conditions, growth of the *A. pleuropneumoniae hlyX* mutant was decreased by 37.3% over a 16-h incubation period compared to the *A. pleuropneumoniae* parent strain (*A. pleuropneu-*

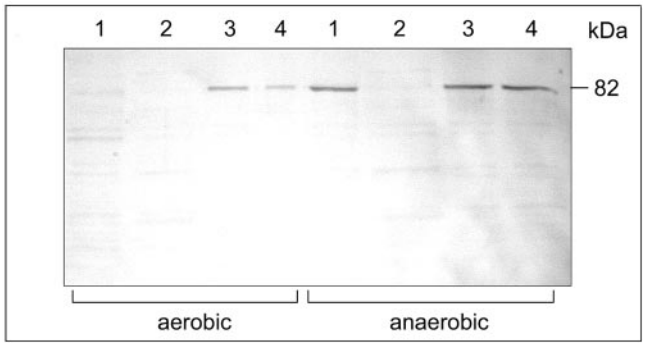

FIG. 2. Expression of the *A. pleuropneumoniae* DmsA protein as assessed by Western blot analysis. Lanes: 1, *A. pleuropneumoniae* AP76; 2, *A. pleuropneumoniae* Δ*hlyX*; 3, *A. pleuropneumoniae* Δ*hlyX* transformed with pHLYX1300; 4, *A. pleuropneumoniae* Δ*hlyX* transformed with pHLYX1301.

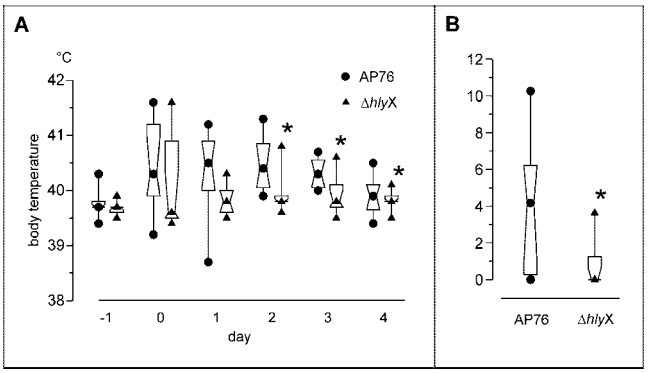

FIG. 3. Virulence studies of *A. pleuropneumoniae* Δ*hlyX* in an aerosol infection model. Symbols: ●, *A. pleuropneumoniae* AP76 wild-type strain (AP76); ▲, *A. pleuropneumoniae* Δ*hlyX* (Δ*dmsA*). The central symbol within the hourglass shape represents the geometric mean, the hinges present the values in the middle of each half of data, and the top and bottom symbols mark the maximum and minimum value. Asterisks denote statistical significance ($P < 0.05$) in the Wilcoxon signed-rank test. (A) Body temperatures of pigs over the course of 6 days, with day 0 marking the day of infection; (B) lung lesion scores assessed according to the method of Hannan et al. (14). Statistical significance ($P < 0.05$) in the Mann-Whitney Test is denoted by an asterisk.

moniae AP76, 21.7 ± 1.15 mg; *A. pleuropneumoniae* Δ*hlyX* 13.6 ± 0.5 mg; $P < 0.01$).

The mutant strain lacked the significant increase in aspartase activity under anaerobic conditions seen in the parent strain (Table 2). Aspartase activity was restored by introducing the *hlyX* gene on plasmid pHLYX1300 but not plasmid pHLYX1301 (Table 2). *A. pleuropneumoniae* Δ*hlyX* showed no expression of DmsA in Western blot analyses; expression was restored by either plasmid pHLYX1300 or pHLYX1301 under anaerobic conditions (Fig. 2).

Virulence studies. *A. pleuropneumoniae* Δ*hlyX* was used in an aerosol infection model and compared to the parent strain in an experiment with 18 German Landrace pigs that were randomly assigned to two groups. The cultures used for infection were grown to an OD_{600} of 0.39 (*A. pleuropneumoniae* parent strain, group 1) and to an OD_{600} of 0.44 (*A. pleuropneumoniae* Δ*hlyX*, group 2) and then diluted 1:30,000 as described previously (4). Pigs were infected in an aerosol chamber that holds four or five animals as described previously (4). Total infection doses aerosolized in 13 ml of diluted liquid culture as calculated from plate counts were 9.6×10^4 CFU for group 1 and 8.1×10^4 CFU for group 2. Aerosol infection led to an increase in body temperature to 40°C or higher in six out of nine animals in group 1 and in four out of nine animals in group 2 on day 1 postinfection. On days 2, 3, and 4 postinfection, the body temperatures in group 2 were significantly lower than in group 1 ($P < 0.05$, Fig. 3A). One animal in group 1 died on day 2 postinfection due to respiratory failure. All remaining animals were sacrificed on day 21 postinfection. At necropsy, six out of nine pigs in group 2 had no macroscopically visible lung lesions compared to two out of eight animals in group 1. The difference in lung lesion scores between the two groups was statistically significant ($P < 0.05$, Fig. 3B). Histological examination revealed no differences in the organization of lesions in both groups.

In group 1, *A. pleuropneumoniae* was isolated as dense to confluent growth from sequesters in all six animals that exhibited lung lesions; however, due to contamination of two samples, quantitative analysis of bacterial counts in lung tissue was successful only in four animals (4.6×10^6 to 4×10^7 per g of tissue). In group 2, *A. pleuropneumoniae* was present in sequestered lung material in only two of the three pigs with lung lesions, at 2.6×10^5 and 3.4×10^5 per g of tissue, respectively. In animals from group 1, *A. pleuropneumoniae* could be isolated from tonsils (six of eight pigs, 6.2×10^4 to 3×10^7 CFU/g of tissue), tracheobronchial lymph nodes (four of eight pigs), and macroscopically unaltered lung tissue (seven of eight pigs). In contrast, no *A. pleuropneumoniae* could be isolated from these tissues in any of the animals in group 2.

Serum samples were obtained 1 week before and 3 weeks after experimental infection. Before the infection, all animals were seronegative. In group 1, seven of eight animals were seropositive; in contrast, in group 2, only the three animals that had lung lesions were serologically positive in the ApxII-ELISA (17; data not shown).

Influence of GSH on aspartase activity. The reporter strain *A. pleuropneumoniae* Δ *aspA*::*luxAB* (15) was used to investigate the influence of GSH on aspartase activity. Liquid cultures were split at an OD_{600} of 0.53. Cultures were diluted 1:2 with 150 mM NaCl, resulting in an OD_{600} of 0.275. After 1 h of incubation, all cultures had reached an OD_{600} of 0.7 to 0.75 with the exception of the culture that had received 12 mM GSH, which only grew to an OD_{600} of 0.36. A gradual increase in luminescence as a result of increased aspartase activity was observed up to a GSH concentration of 1,200 μM, at which a threefold increase in activity was seen, whereas the culture that received 12 mM GSH showed no luciferase activity (Fig. 4).

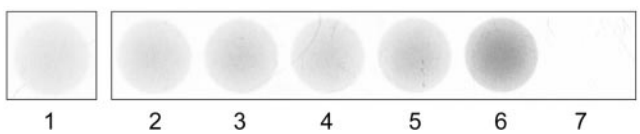

FIG. 4. Influence of GSH on HlyX-induced activation of *aspA* transcription in a luciferase assay using *A. pleuropneumoniae* Δ*aspA*::*luxAB*. Lanes: 1, NaCl control; 2, 1.2 μM GSH; 3, 12 μM GSH; 4, 120 μM GSH; 5, 600 μM GSH; 6, 1,200 μM GSH; 7, 12 mM GSH.

Upon addition of 80 μM oxidized GSH, which is the maximum physiological concentration that was measured in the murine lung model (8), no increase in luciferase activity could be induced (data not shown).

DISCUSSION

In *E. coli* the FNR protein, a global response regulator containing four iron-sulfur clusters, upregulates the expression of a number of genes involved in anaerobic metabolism, such as the gene coding for DMSO reductase, and simultaneously represses genes involved in aerobic metabolism, such as the gene coding for cytochrome oxidase (6). In *A. pleuropneumoniae* the FNR homologue HlyX, which is able to complement *fnr* deletions in *E. coli*, has been identified (18). As we had shown in previous studies, genes containing putative HlyX binding motifs are expressed by *A. pleuropneumoniae* under the influence of bronchoalveolar lavage fluid from infected pigs, and deletion of these genes can cause attenuation of the organism (2, 15). In addition, an analysis of genes expressed in necrotic porcine lung tissue revealed the expression of DMSO reductase and several other genes involved in anaerobic metabolism under these conditions (1). This repeated isolation of presumably HlyX-regulated genes led to the hypothesis that deletion of this global anaerobic regulator would inhibit colonization and/or persistence in *A. pleuropneumoniae* if it was indeed involved in the regulation of the genes we identified. Since neither the regulation of *A. pleuropneumoniae* genes by HlyX nor the impact of the deletion of a global anaerobic regulator on the virulence of a respiratory tract pathogen had been investigated to date, we constructed a *hlyX* deletion mutant and examined it both in vitro and in vivo in an aerosol infection experiment.

The isogenic mutant *A. pleuropneumoniae* Δ*hlyX* shows a markedly reduced growth rate under anaerobic conditions in vitro, which implies that HlyX regulation is important but not essential for adaptation of *A. pleuropneumoniae* to anaerobiosis in culture. Our finding that both DMSO reductase expression and induction of aspartase activity are abolished in the *hlyX*-negative mutant supported our hypothesis that expression of these genes is regulated by HlyX (Fig. 2 and Table 2). In order to confirm that the effects we observed were specific for *hlyX*, we set up a complementation experiment reintroducing a plasmid-encoded *hlyX* gene into *A. pleuropneumoniae* Δ*hlyX*; in plasmid pHLYX1300 the *hlyX* gene is positioned in the orientation of transcription initiated by the plasmid-encoded *sulII* promoter, and in pHLYX1301 it is in the opposite orientation. Both DMSO expression and induction of aspartase activity under anaerobic conditions were restored in pHLYX1300 transformants. The finding that pHLYX1301 transformants also had a detectable DmsA expression could be explained by the incidental formation of a fusion promoter in plasmid pHLYX1301 that is able to initiate transcription. Sequence differences between the HlyX binding sites of *dmsA* (TTGAT—ATCAG) and *aspA* (GTGAT—ATCAC) could be responsible for the absence of this effect in the Δ*aspA* mutant. In addition, Western blot analysis may be more sensitive than the aspartase assay in the detection of the fusion promoter activity. The faint bands that are visible under aerobic conditions for the complemented strains may be due to the plasmid-encoded constitutive expression of HlyX.

In the challenge experiment with the *A. pleuropneumoniae* parent strain (group 1) and *A. pleuropneumoniae* Δ*hlyX* (group 2), we observed a significantly reduced virulence of the mutant strain. The finding that six of nine animals in group 2 had no macroscopically visible lung lesions and no detectable antibody titers implied that *A. pleuropneumoniae* Δ*hlyX* was clearly reduced in its ability to colonize, a prerequisite for inflicting lung damage. In contrast to the findings in group 2, six of the surviving eight animals in group 1 had severe lesions; additionally, one animal in this group died on day 2 postinfection.

The reduced colonizing and persisting ability of *A. pleuropneumoniae* Δ*hlyX* was confirmed by the complete lack of reisolation from tonsils and healthy lung epithelium 21 days postinfection; in contrast, the majority of animals challenged with the parent strain (group 1) still harbored the organisms in these tissues. Further, an *hlyX* deletion appears to also hamper long-term survival inside sequestered lung tissue, since *A. pleuropneumoniae* Δ*hlyX* was completely absent from the lung lesions of one animal in group 2, and the number of CFU per gram of material from the other two animals was 1 to 2 logs below the numbers found in animals in group 1. This difference likely is the result of ineffective adaptation of *A. pleuropneumoniae* Δ*hlyX* to anaerobic conditions in sequestered lung tissue and tonsils, and derepression of FNR-regulated genes involved in aerobic metabolism may add to this effect by causing the bacterium to waste energy. Together, these results confirm and extend the observations we made with mutant strains lacking DMSO reductase and aspartase activity (2, 15) and clearly show that genes under transcriptional control of the anaerobic regulator HlyX encode virulence-associated proteins required for bacterial survival in the presumably aerobic environment on the respiratory epithelium.

In an initial attempt to solve this apparent contradiction, we hypothesized that an airway antioxidant such as GSH or nitrogen species might be responsible. GSH is secreted by respiratory epithelial cells of a number of mammals including pigs and humans (7, 8, 34) as a primary line of defense against reactive oxygen or nitrogen species might be responsible. This hypothesis was particularly intriguing since GSH has recently been shown to be upregulated in the murine lung during infection with *Pseudomonas aeruginosa* (7, 8, 34). Further, GSH, on the one hand, reduces the redox potential of the epithelial lining fluid (ELF), a signal which has been shown to induce expression of FNR-regulated genes in *E. coli* (31). On the other hand, GSH has been shown to stabilize the iron-sulfur clusters required for the DNA-binding of FNR (29). Our finding that a concentration of 1,200 μM GSH induces luciferase expression in *A. pleuropneumoniae* Δ*aspA*::*luxAB* in culture supports this hypothesis, since this concentration has been calculated to be present in the murine lung (8); in addition, oxidized glutathione at a concentration of 80 μM, the maximum concentration observed in the murine lung (8), failed to achieve the same effect, which demonstrates that the effect we observed is specific for the reduced form of glutathione. The observed bactericidal activity of a GSH concentration of 1.2 mM is supported by reports of other groups investigating the role of GSH in the respiratory tract (13, 32). Therefore, these initial results imply that GSH is one of the factors in ELF that is sensed by *A. pleuropneumoniae*, thereby causing an HlyX-induced expression of virulence-associated proteins. Whether GSH acts by reducing oxygen

tension or by direct interaction with the iron-sulfur clusters of HlyX and, likewise, which other factors in ELF might be recognized by the pathogen remains to be determined.

The fact that *A. pleuropneumoniae* Δ*hlyX* is still virulent and able to persist in sequestered lung tissue further demonstrates that HlyX is not essential for virulence or adaptation to oxygen-reduced conditions and therefore implies that other, as-yet-unidentified regulators are able to partially compensate for the loss of HlyX function. One possible candidate, the global regulator ArcA has recently been linked to virulence in *Haemophilus influenzae* and *Vibrio cholerae* (23, 27). ArcA has not been characterized in *A. pleuropneumoniae*; however, genomic sequence data available for *A. pleuropneumoniae* at http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi? under the sequence ID NZ_AACK01000005 reveal a putative response regulator protein that is 73% identical to the ArcA protein of *H. influenzae* (accession number NP_439045). Whether this putative ArcA protein plays a role in *A. pleuropneumoniae* virulence and, if so, how coordination of HlyX and ArcA regulation of virulence-associated genes occurs in the different compartments affected by an *A. pleuropneumoniae* infection (tonsils, sequestered lung, and intact respiratory epithelium) remains to be determined.

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