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 (Δθap), 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; Merek, Darmstadt, Germany), t-glutamine (100 μg/ml; Serva, Heidelberg, Germany), t-cysteine-hydrochloride (260 μg/ml; Sigma-Aldrich), t-cysteine-dihydrochloride (10 μg/ml; Sigma-Aldrich), dextrose (1 mg/ml), and Tween 80 (0.1%) on Columbia Sheep

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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 (OD600) of 0.3. The culture was chilled on ice for 15 min, and bacteria were harvested by centrifugation. 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.

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

<table>
<thead>
<tr>
<th>Strains</th>
<th>Characteristics</th>
<th>Source and/or references</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
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<td></td>
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<tr>
<td><em>E. coli</em> B2155</td>
<td>thrB1004 pro thi strA hsdS lacZΔM15 (F’ lacZΔM15 traD36 proA+ proB+)</td>
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<tr>
<td><em>E. coli</em> DH5αF’</td>
<td>F’ endA1 hsdR17 (rK– mK–) supE44 thi– recA1 gyrA (Nal’) relA1 ΔlacZYA- argF)U169 deoR [6808lacΔ(lacZ)M15]</td>
<td>21</td>
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<tr>
<td><strong>A. pleuropneumoniae</strong> AP76</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>A. pleuropneumoniae</strong> ΔhlyX</td>
<td>Unmarked <em>A. pleuropneumoniae</em> mutant carrying luxAB as a transcriptional fusion in the <em>aspA</em> gene</td>
<td>This study</td>
</tr>
<tr>
<td><strong>A. pleuropneumoniae</strong> ΔasPA::luxAB</td>
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**Plasmids**

<table>
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<th>Plasmids</th>
<th>Characteristics</th>
<th>Source and/or references</th>
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<tbody>
<tr>
<td>pCR2.1-TOPO</td>
<td>Topoisomerase I enhanced <em>E. coli</em> cloning vector</td>
<td>TOPO TA Cloning, Stratagene, La Jolla, CA Accession no. AJ866288 (5)</td>
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<tr>
<td>pBluescript SK(+)</td>
<td>3.0-kb cloning vector; Ap’</td>
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<tr>
<td>pEMOC2</td>
<td>Transconjugation vector based on pBluescript SK with mobRP4, polycloning site, Cm’, and transcriptional fusion of the <em>omlA</em> promoter with the <em>sacB</em> gene</td>
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<td>pHLYX810</td>
<td>2,338-bp PCR product of primers oHLYX5 and oHLYX6, containing the <em>hlyX</em> ORF, cloned into pCR 2.1-TOPO</td>
<td>This study</td>
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<td>pHLYX110</td>
<td>ApaI/NotI fragment carrying the <em>hlyX</em> ORF from pHLYX810, ligated into pBluescript SK(+)</td>
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<td>pHLYX111</td>
<td>Deletion of an 883-bp fragment between the BglII and XcmI restriction sites of pHLYX110</td>
<td>This study</td>
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<tr>
<td>pHLYX701</td>
<td>ApaI/NotI fragment from pHLYX111, ligated into pEMOC2</td>
<td>This study</td>
</tr>
<tr>
<td>plS88</td>
<td>Broad-host-range shuttle vector from <em>Haemophilus ducreyi</em>; Sm’ Km’</td>
<td>33</td>
</tr>
<tr>
<td>pHLYX1300</td>
<td>PCR product generated from primers oHLYX9 and oHLYX10 containing <em>hlyX</em> ORF, ligated into plasmid pLS88 after MfeI restriction in a 3’–3’ orientation with respect to the vector-derived sulII promoter</td>
<td>This study</td>
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<tr>
<td>pHLYX1301</td>
<td>PCR product generated from primers oHLYX9 and oHLYX10 containing <em>hlyX</em> ORF ligated into plasmid pLS88 after MfeI restriction in a 3’–5’ orientation with respect to the vector-derived sulII promoter</td>
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**Primers**

<table>
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<th>Primers</th>
<th>Characteristics</th>
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<tr>
<td>oHLYX5</td>
<td>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 <em>hlyX</em> start codon</td>
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<td>oHLYX6</td>
<td>5’-TCG CGG CCG CCA ACG TGA GAG CTT CGT TCA-3’; reverse primer containing an NotI restriction site, situated 626 bp downstream of the <em>hlyX</em> ORF</td>
<td>This study</td>
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<td>oHLYX7</td>
<td>5’-TCC GAA ACC GGA TAA TTC AC-3’; forward primer situated 507 bp upstream of the <em>hlyX</em> start codon</td>
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<td>oHLYX8</td>
<td>5’-AGC GAA AGG GTT AAT CAG CA-3’; reverse primer situated 228 bp downstream of the <em>hlyX</em> ORF</td>
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<td>oHLYX9</td>
<td>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 <em>hlyX</em> start codon</td>
<td>This study</td>
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<td>oHLYX10</td>
<td>5’-CTT ACA ATT GGC CCT ATA CCG TCA GTT G-3’; reverse primer containing an MfeI restriction site situated 4 bp downstream of the <em>hlyX</em> ORF</td>
<td>This study</td>
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*Erm’, erythromycin resistance; Te’, tetracycline resistance; Km’, kanamycin resistance; Str’, streptomycin resistance; Sm’, sulfonamide resistance; Km’, 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 were 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, 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 (OD600) of 0.3. The culture was chilled on ice for 15 min, and bacteria were harvested by centrifugation.
cine [inactivated]) was used to assess the clinical condition of each individual
assigned to the different groups and cared for in accordance with the principles
clinically healthy pigs (German Landrace) 7 to 9 weeks of age were randomly
A. pleuropneumoniae
aerosol infection model previously described (4);

Cloning of the A. pleuropneumoniae hlyX gene and construction of an isogenic
deletion mutant. The hlyX gene (GenBank accession no. M80712) of A. pleuropneu-
moniae was amplified on a 2.383-bp fragment from genomic A. pleuropneu-
moniae AP76 DNA by using primers oHLYX5 and oHLYX6 (Table 1 and Fig. 1),
which contained Apal and NotI restriction endonuclease sites, respectively.

Complementation of A. pleuropneumoniae ΔhlyX. Plasmids pHLYX1300 and
pHLYX1301 were constructed by cloning the 746-bp MseI-restricted PCR prod-
uct generated by primers oHLYX9 and oHLYX10 (Table 1 and Fig. 1), con-
taining the entire hlyX open reading frame (ORF) without the putative promoter
region, into EcoRI-restricted plasmid pLS88 (33) and electroporated into A. pleuro-
moniae ΔhlyX. In plasmid pHLYX1300 the orientation of the hlyX gene is in
the orientation of transcription initiated by the vector-derived nusI promoter;
in pHLYX1301 it is located in the opposite orientation.

Aspartase test was performed by determination of fumarate formation as de-
scribed previously (5, 20) resulting in A. pleuropneumoniae ΔhlyX.

Wild type .......................................................... 191 ± 24
Mutant ΔhlyX ..................................................... 106 ± 21
Mutant ΔhlyX + pHLYX1300 ................................ 202 ± 25
Mutant ΔhlyX + pHLYX1301 ............................. 104 ± 13

* Arithmetic mean of results from three independent experiments. The rela-
tive activity under aerobic conditions is 100%

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 at a

RESULTS

Construction and functional characterization of the isogenic
mutant A. pleuropneumoniae ΔhlyX 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. pleuropneu-
moniae 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 com-
pared to the A. pleuropneumoniae parent strain (A. pleuropneu-
lesions, at $2.6 \times 10^5$ and $3.4 \times 10^5$ per g of tissue, respectively. In animals from group 1, *A. pleuropneumoniae* could be isolated from tonsils (six of eight pigs, $6.2 \times 10^4$ to $3 \times 10^5$ 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 was seen, whereas the culture that received 12 mM GSH showed no luciferase activity (Fig. 4).

![Figure 2](image1.png)


![Figure 3](image2.png)

**FIG. 3.** Virulence studies of *A. pleuropneumoniae ΔhlyX* in an aerosol infection model. Symbols: ●, *A. pleuropneumoniae* AP76 wild-type strain (AP76); ▲, *A. pleuropneumoniae* ΔhlyX (ΔdmsA). 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.

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 ($4.6 \times 10^9$ to $4 \times 10^7$ 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 $2.6 \times 10^5$ and $3.4 \times 10^5$ per g of tissue, respectively. In animals from group 1, *A. pleuropneumoniae* could be isolated from tonsils (six of eight pigs, $6.2 \times 10^4$ to $3 \times 10^5$ 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.

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**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 mM, at which a threefold increase in activity was seen, whereas the culture that received 12 mM GSH showed no luciferase activity (Fig. 4).
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 ΔhlyX* 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 ΔhlyX*; 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—ATCAC) 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 ΔhlyX* (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 ΔhlyX* 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 ΔhlyX* 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 ΔhlyX* 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 ΔhlyX* 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 ΔaspA::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 Actinobacillus pleuropneumoniae ΔhlyX 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 Actinobacillus pleuropneumoniae; however, genomic sequence data available for Actinobacillus pleuropneumoniae at http://www.ncbi.nlm.nih.gov/utslib/genom_table.cgi under the sequence ID NZ_AACK01000005 reveal a putative response regulator protein that is 75% identical to the ArcA protein of H. influenzae (accession number WP_4390945). Whether this putative ArcA protein plays a role in Actinobacillus pleuropneumoniae virulence and, if so, how coordination of HlyX and ArcA regulation of virulence-associated genes occurs in the different compartments affected by an Actinobacillus pleuropneumoniae infection (tonsils, sequestered lung, and intact respiratory epithelium) remains to be determined.

ACKNOWLEDGMENTS

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