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ohr, Encoding an Organic Hydroperoxide Reductase, Is an In Vivo-Induced Gene in *Actinobacillus pleuropneumoniae*

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Actinobacillus pleuropneumoniae **is the causative agent of porcine pleuropneumonia, a disease characterized by pulmonary necrosis and hemorrhage caused in part by neutrophil degranulation. In an effort to understand the pathogenesis of this disease, we have developed an in vivo expression technology (IVET) system to identify genes that are specifically up-regulated during infection. One of the genes that we have identified as being induced in vivo is *ohr*, encoding organic hydroperoxide reductase, an enzyme that could play a role in detoxification of organic hydroperoxides generated during infection. Among the 12 serotypes of *A. pleuropneumoniae*, *ohr* was found in only serotypes 1, 9, and 11. This distribution correlated with increased resistance to cumene hydroperoxide, an organic hydroperoxide, but not to hydrogen peroxide or to paraquat, a superoxide generator. Functional assays of Ohr activity demonstrated that *A. pleuropneumoniae* serotype 1 cultures, but not serotype 5 cultures, were able to degrade cumene hydroperoxide. In *A. pleuropneumoniae* serotype 1, expression of *ohr* was induced by cumene hydroperoxide, but not by either hydrogen peroxide or paraquat. In contrast, an *ohr* gene from serotype 1 cloned into *A. pleuropneumoniae* serotype 5 was not induced by cumene hydroperoxide or by other forms of oxidative stress, suggesting the presence of a serotype-specific positive regulator of *ohr* in *A. pleuropneumoniae* serotype 1.**

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a disease characterized by massive lung necrosis and pulmonary hemorrhage. This necrosis is due in part to the influx of host immune cells and the release of neutrophil lysosomal contents that include oxygen radicals, which can destroy the invading bacteria as well as the host tissue (27). In an effort to understand the pathogenesis of this respiratory disease and the effect of the host immune response on *A. pleuropneumoniae*, we have developed an in vivo expression technology (IVET) system to identify genes that are expressed during infection but not during growth in vitro on laboratory media (13). One of the genes that we have identified with this selection shows homology to *ohr* (organic hydroperoxide reductase), which has been described previously for *Xanthomonas campestris* (24), *Pseudomonas aeruginosa* (29), *Enterococcus faecalis* (33), and *Bacillus subtilis* (12). Ohr has been implicated in resistance to and detoxification of the organic peroxides, such as cumene hydroperoxide (CHP) (24, 29).

Bacteria are frequently exposed to reactive oxygen species during the course of infection. Oxygen radicals in the form of superoxides, hydrogen peroxides, and organic hydroperoxides can result from release of lysosomal contents within inflammatory cells or can be generated by bacterial cellular metabolism (3, 38). During infection of the porcine lung, *A. pleuropneumoniae* is exposed to oxygen radicals in the form of superoxides and peroxides generated by the neutrophil oxidative burst (27). A third class of oxygen radicals, organic hydroperoxides, can be generated either directly within the phagosome or as a consequence of oxygen radicals interacting with the bacterial cell membrane (reviewed in the work of Miller and Britigan [23]). To survive and protect its cellular metabolism within this dangerous milieu, *A. pleuropneumoniae* may require enzymes capable of inactivating these oxygen species (20).

A. pleuropneumoniae has been shown previously to contain catalase and two distinct superoxide dismutases, SodA and SodC, which can relieve a portion of the oxidative stress that occurs during infection (21). Enzymes that could detoxify the third category of oxidative stress reagents, the organic hydroperoxides, have not been previously identified in *A. pleuropneumoniae*. In this work, we identify another potentially protective gene, *ohr*, which is specifically induced during infection and produces a protein that is capable of detoxifying organic peroxides encountered by *A. pleuropneumoniae* during infection of the porcine lung.

MATERIALS AND METHODS

Bacterial strains. The *A. pleuropneumoniae* strains that were used in this study and the plasmids propagated in these strains are listed in Table 1. *A. pleuropneumoniae* strains were cultured at 35°C on either brain heart infusion (BHI) (Difco, Detroit, Mich.) or heart infusion (Difco) medium supplemented with V factor (β-NAD) added at 10 μg/ml (BHIV or HIV, respectively). Riboflavin, when needed for maintenance of APP233, was added at 200 μg/ml. Medium was supplemented with ampicillin at 50 μg/ml to propagate plasmids or at 20 μg/ml to recover *A. pleuropneumoniae* from porcine lungs and to select for transformants after electroporation. *Escherichia coli* strain XLI-Blue mRF' (Stratagene, La Jolla, Calif.) was used for construction and propagation of plasmids. *E. coli* was cultured on Luria-Bertani medium (Difco) supplemented with ampicillin at 100 μg/ml.

Molecular manipulations. Genomic DNA from *A. pleuropneumoniae* was prepared according to the lysis-proteinase K method described in the work of Silhavy (36). Plasmid DNA was purified using Qiagen spin columns (Qiagen Inc., Valencia, Calif.). DNA-modifying enzymes were obtained from Roche (Roche Molecular Biochemicals, Indianapolis, Ind.) and used according to the manufacturer's specifications.

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

Strain or plasmid	Characteristic(s)	Source (reference)
Strains		
APP225	*A. pleuropneumoniae* ATCC 27088, serotype 1A, passaged through pigs	Fuller et al. (15)
APP227	*A. pleuropneumoniae* ISU178, a serotype 5 field isolate, passaged through pigs	Fuller et al. (14)
APP233	A double-crossover riboflavin auxotroph of APP225	Fuller et al. (15)
ATCC 27089	*A. pleuropneumoniae* serotype 2	ATCC[a]
ATCC 27090	*A. pleuropneumoniae* serotype 3	ATCC
ATCC 33378	*A. pleuropneumoniae* serotype 4	ATCC
ATCC 33590	*A. pleuropneumoniae* serotype 6	ATCC
ISU53	*A. pleuropneumoniae* serotype 7	V. Rapp, Iowa State University
405	*A. pleuropneumoniae* serotype 8	R. Nielsen, Denmark
CVJ1326	*A. pleuropneumoniae* serotype 9	R. Nielsen, Denmark
13039	*A. pleuropneumoniae* serotype 10	R. Nielsen, Denmark
56513	*A. pleuropneumoniae* serotype 11	R. Nielsen, Denmark
1096	*A. pleuropneumoniae* serotype 12	R. Nielsen, Denmark
Plasmids		
pTF86	*A. pleuropneumoniae* IVET vector containing promoterless *luxAB* and *ribBAH* genes downstream of a unique *Bam*HI cloning site	Fuller et al. (13)
piviK	pTF86 containing 801-bp insert with 294 bp of *ohr* in *Bam*HI site	This study
pGEM-T	Ap^r TA cloning vector for PCR fragments	Promega
pinKE	pGEM-T containing 2.5-kb insert with *ohr* inverse PCR fragment	This study
pGZRS18	Ap^r *A. pleuropneumoniae-E. coli* shuttle vector	West (44)
pGeohr	pGZRS18 containing full-length *ohr* gene with 360 bp of upstream sequence	This study

[a] ATCC, American Type Culture Collection, Manassas, Va.

Preparation of electrocompetent *A. pleuropneumoniae* serotype 1 and electroporation of these cells with plasmid DNA prepared from either *E. coli* or *A. pleuropneumoniae* serotype 1 was performed as previously described (15). *A. pleuropneumoniae* serotype 5 (14) was made competent by the method of Ward et al. (43) and electroporated using the same electroporation conditions as those for serotype 1 but using plasmid prepared from *E. coli*. *E. coli* transformation was performed by the Hanahan method (16).

Southern blotting. Genomic DNA from all 12 *A. pleuropneumoniae* serotypes (Table 1) was digested to completion using *Eco*RI and separated on a 0.8% agarose gel. DNA fragments were transferred to a Nytran (Schleicher & Schuell, Keene, N.H.) membrane by the method of Southern (35). The membrane was hybridized with a digoxigenin-labeled probe generated by PCR amplification of the complete *ohr* gene using primers (MM150, 5′-GACAAGAATTCAACAAG GACAATATTATG-3′, and MM151, 5′-CCTAAATCGTCCCAGATCTGGTA GG-3′) that flank the open reading frame (ORF) (Roche PCR DIG synthesis kit). For high-stringency hybridization, blots were incubated for 16 h at 42°C with the probe diluted in a hybridization buffer that contained 50% formamide, 5× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate), and 2% blocking reagent (Roche). High-stringency washes were performed at 68°C with 0.1× SSC–0.1% sodium dodecyl sulfate. For low-stringency hybridization, blots were incubated for 16 h at 42°C with the probe diluted in an aqueous solution containing 6× SSC and 1% blocking reagent, with no formamide added. Low-stringency blots were washed for 10 min at room temperature with 2× SSC–0.1% sodium dodecyl sulfate. Hybridizing bands were detected using alkaline phosphatase-tagged antidigoxigenin and the CDP* chemiluminescent substrate (Roche).

Infection model. The infection of pigs with either APP233/piviK or APP233/pTF86 was performed as previously described (13). Briefly, each clone was inoculated into HIV broth containing 200 μg of riboflavin/ml, 50 μg of ampicillin/ml, and 5 mM calcium chloride and grown at 35°C to an optical density at 520 nm (OD_{520}) of 0.8. The bacteria were washed with saline and resuspended in 10 ml of saline containing 200 μg of riboflavin/ml at a final concentration of 7×10^8 CFU. The bacterial suspension was inoculated, via an endotracheal catheter, into the lungs of 6-week-old pigs. The pigs were monitored for development of clinical signs of pleuropneumonia as previously described (18) and in conjunction with animal use approval. Sterile lung samples were collected for bacterial isolation and photography following necropsy. Lung samples were examined for in situ luciferase activity by using a Hamamatsu C1966 photonic microscope system (13). All experimental protocols for animal experiments were reviewed by the Michigan State University All-University Committee on Animal Use and Care, and all procedures conformed to university and U.S. Department of Agriculture regulations and guidelines.

Cloning of the intact *ohr* gene. An inverse PCR technique was designed to clone the intact *ohr* gene (28). A 2.5-μg quantity of *A. pleuropneumoniae* serotype 1 genomic DNA was digested to completion with *Eco*RI, followed by a self-ligation using T4 DNA ligase (Roche) to form closed circular fragments. PCR amplification with AmpliTaq (Roche) was performed with 0.04 μg of ligated DNA using primers (MM139, 5′-AACCAAGTGAACCGTCATCTACTC-3′, and MM140, 5′-GTGGCAAAGTCGGCACAAACC-3′) designed from the known sequence of the *iviK* clone, which contained the promoter region and partial ORF of the *ohr* gene. These primers bound within the coding region of *ohr* and were oriented to PCR amplify the flanking regions. The resulting 2.5-kb PCR fragment was isolated from an agarose gel using the Qiaex II kit (Qiagen) and was cloned into the pGEM-T vector (Promega, Madison, Wis.) to form pinKE. This clone was sequenced using an ABI100 Model 377 automated sequencer (Applied Biosystems, Foster City, Calif.) and a primer (MM141, 5′-CT GTAGGCGTGGGAATCGGTC-3′) internal to the known *ohr* sequence. A complete ORF was identified by comparison of the sequence obtained from the *iviK* clone and the sequence that resulted from the inverse PCR clone pinKE. The complete *ohr* ORF with the upstream promoter region was amplified from *A. pleuropneumoniae* serotype 1 genomic DNA using *Pfu* polymerase (Promega) and a primer pair (MM138, 5′-GGCTACGAAATATTGGACACG-3′, and MM151) that binds 360 bp upstream of the start codon and 50 bp downstream of the stop codon. The PCR product was cloned into *Sma*I-cut pGZRS18 to form pGeohr (44). This plasmid was transformed into both *A. pleuropneumoniae* serotype 1 and *A. pleuropneumoniae* serotype 5.

Oxidative stress growth inhibition assay. Hydrogen peroxide (Sigma, St. Louis, Mo.), CHP (Sigma), and the superoxide generator paraquat (Sigma) were used as oxidative stress reagents. Disk inhibition assays were used to analyze bacterial sensitivity to these reagents (38). Briefly, 100 μl of an overnight bacterial liquid culture was added to 3 ml of BHIV top agar (0.7%) and poured onto BHIV plates. Filter paper disks (10 mm, Whatman no. 1; Whatman Paper Ltd., Maidstone, England) saturated with 10 μl of 0.88 M hydrogen peroxide, 200 mM CHP, or 0.074 M paraquat were placed onto the hardened top agar (38). Diameters of the zones of growth inhibition were recorded after 22 h of incubation at 35°C under 5% CO_2. Statistical analysis of zone diameter significance within each treatment group for serotypes containing *ohr* compared to serotypes lacking the gene was evaluated by a two-tailed Student *t* test. The Analysis ToolPak in Microsoft Excel (2000) was used to perform the *t* test under homoscedastic and heteroscedastic conditions.

Oxidative stress and measurement of *lux* expression. Induction of oxidative stress in broth cultures was performed as follows. *A. pleuropneumoniae* strains were grown in 25 ml of BHIV broth containing 50 μg of ampicillin/ml, at 35°C and with shaking at 150 rpm, to an OD_{520} of 0.8 and were then dispensed into a

96-well microtiter plate in 200-μl aliquots. This was followed by a 30-min period of incubation at 35°C under 5% CO_2 prior to addition of the stress reagent to allow for acclimation. For stress induction, stress reagents were added to a final sublethal concentration of 125 μM, 300 μM, or 1 mM CHP; 56 μM hydrogen peroxide; or 50 μM paraquat (38). Aliquots were taken for luminometric assays and primer extension at designated intervals.

Quantitative luciferase assays were performed using a Turner Model 20e luminometer as previously described (13). Briefly, 20 μl of culture was mixed with 20 μl of *N*-decyl aldehyde in a polypropylene luminometer cuvette. The sample was read in full integral, autoranging mode with a predelay of 0 s, a delay of 10 s, and an integration time of 30 s. The *N*-decyl aldehyde substrate was made by sonicating 20 mg of Essentially Fatty Acid Free Bovine Serum Albumin (Sigma)/ml with 1 μl of *N*-decyl aldehyde/ml in a sonicating water bath at room temperature. Luminometer readings were normalized to relative light units (RLU) per OD_{520} unit.

Functional analysis of Ohr activity. An assay to evaluate the degradation of CHP was adapted from procedures developed by Dringen et al. (9) and Ochsner et al. (29). *A. pleuropneumoniae* was grown in BHIV broth to early stationary phase (OD_{520} = 0.8 to 1.0) and diluted with fresh prewarmed medium. CHP was added to a final concentration of either 0, 125, 300, or 600 μM CHP. Residual CHP concentrations were determined at 5-min intervals by a xylenol orange-iron reaction. At each time point, 100 μl of the culture was pelleted and 20 μl of the cell-free supernatant was added to 80 μl of 25 mM sulfuric acid in a 96-well microtiter plate. When all samples had been collected, 100 μl of freshly prepared reaction buffer containing 200 μM xylenol orange (Sigma), 200 μM ammonium ferrous sulfate (Sigma), and 25 mM sulfuric acid was added to each sample. After 10 min of incubation at room temperature, absorbance was read at 540 nm using a Bio-Tek ELISA Plate Reader Model EL310 (Bio-Tek Instruments, Inc., Winooski, Vt.). Concentrations of CHP in each sample were determined by comparison to a CHP standard curve performed at the time of each assay. Ohr activity was measured as micromoles of CHP degraded per minute.

To evaluate the induction of Ohr activity by CHP, CHP was added to 1 ml of the freshly diluted culture at a final concentration of 0, 125, or 300 μM, and this mixture was held without shaking for 30 min at 35°C. A sample was collected to determine the residual CHP concentration. Fresh CHP was added, and Ohr activity was assayed as described above.

Primer extension. Primer extension of the *ohr* gene was performed as previously described (8) using a primer (MM220, 5′-CGAGTATGACCATCACGA CCGCCAACTGC-3′) that bound 30 bp into the *ohr* coding region. Bacteria were incubated for 30 min in a 96-well microtiter plate under inducing conditions with 1 mM CHP, and without CHP as a noninduced control. The mRNA was isolated using a hot-phenol extraction method (45). For reverse transcription, 10 μg of RNA was incubated with 1 pM [^{32}P]ATP (Amersham Pharmacia Biotech, Piscataway, N.J.)-labeled primer and avian myeloblastosis virus reverse transcriptase (Promega) for 1 h at 42°C. The samples were separated on an 8% denaturing polyacrylamide gel along with a ^{35}S-dATP (Amersham) sequencing ladder. The sequencing ladder was prepared using the Sequenase version 2.01 kit (Amersham) and the same primer that was used for primer extension (8).

Nucleotide sequence accession number. The sequence reported in this paper has been submitted to GenBank and assigned accession no. AF395877.

RESULTS

Identification of *ohr* as an in vivo-induced gene. We previously developed an IVET screen to identify genes from *A. pleuropneumoniae* that were induced during infection of the porcine lung but had minimal expression during in vitro growth on laboratory media (13). This IVET system utilized a promoter trap vector (pTF86) with a cloning site for genomic DNA fragments upstream of promoterless copies of the *luxAB* and *ribBAH* genes. A library of random genomic DNA fragments from *A. pleuropneumoniae* serotype 1 was cloned into the pTF86 vector and subsequently electroporated into APP233, a virulent *A. pleuropneumoniae* serotype 1 strain that is unable to produce riboflavin due to a directed mutation within the *ribGBAH* operon (15). When a functional promoter was placed in the cloning site of pTF86, riboflavin was produced and complemented the riboflavin deficiency of the host strain, APP233, restoring full virulence. Without a functional promoter, riboflavin was not produced and APP233 failed to survive and cause disease (15). Thus, the initial portion of the IVET procedure utilized infection of the natural host to select clones containing promoters that were expressed during infection. Clones containing functional promoters were isolated from the pig lung and examined for in vitro expression of the *luxAB* genes both quantitatively and qualitatively. Clones that had in vitro expression levels in APP233 that were ≤200 RLU/OD_{520} unit were identified as *ivi* (in vivo-induced) clones.

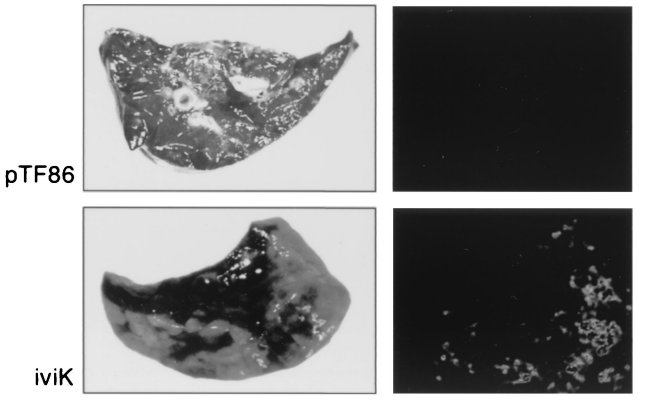

FIG. 1. Pulmonary damage and in vivo *lux* expression resulting from infection of a swine lung with either APP233/pTF86 (top) or APP233/piviK (bottom). For each sample, on the left is an incidental light photograph of the lung specimen. On the right is a corresponding photograph, taken by photonic camera, showing bioluminescence of the same lung specimen. In the absence of a promoter, APP233/pTF86 showed no lung damage and no *lux* expression. In the presence of the piviK plasmid, significant lung damage was apparent and bioluminescence was seen.

Forty-two unique *ivi* clones were identified during this selection. One of these clones, *iviK*, contained an 801-bp insert that included a partial ORF of 98 amino acids fused to *luxAB*, as well as 507 bp of upstream noncoding sequence. When this ORF, which contained a start codon but lacked a stop codon, was used to search microbial sequence databases, it showed 56% similarity to Ohr from *X. campestris*, an enzyme responsible for protection against organic peroxides (24).

To confirm the in vivo induction of the *iviK* promoter, a pig was infected intratracheally with 7×10^8 CFU of APP233/piviK to monitor development and progression of the pulmonary disease. We have previously demonstrated that APP233 alone is avirulent at doses as high as 5×10^9 CFU in this animal infection model (13, 15). Within 6 h, the infected pig developed an increased respiratory rate and fever and showed depression and anorexia. The disease progressed to severe dyspnea by 9 h postinfection. At necropsy, 90 to 100% of the right lung lobes and the accessory lobe showed edema, hemorrhage, congestion, and regions of necrosis. These symptoms are consistent with peracute pleuropneumonia. A portion of the right caudal lung lobe from this pig was photographed by visible light camera and by photonic camera (Fig. 1). The visible light picture shows regions of severe necrosis and hemorrhage. The photonic camera picture of this same region of lung shows *lux* expression at the edges of this necrotic tissue, which is the region of active infection. In contrast to infection by this *iviK* clone, we have previously shown that infection with

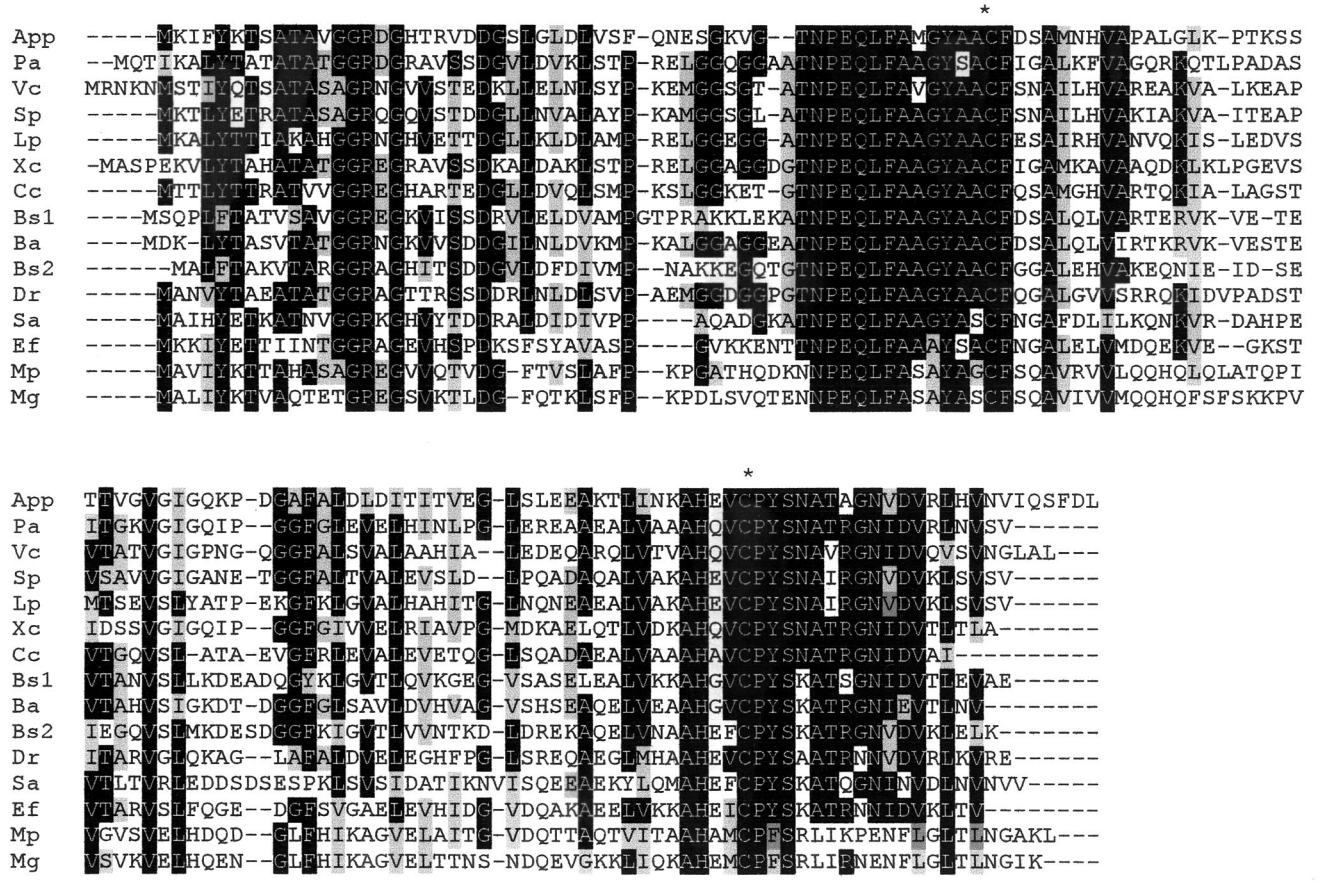

FIG. 2. Protein sequence alignment of putative Ohr proteins from 14 bacterial species aligned with Boxshade v3.31 (http://biophysics.med.jhu.edu/prog/boxshade/PC__and__MAC/win16.zip) and ClustalX (41). Black-shaded regions indicate residues that are identical in the majority of species. Gray-shaded regions indicate residues that are functionally conserved in the majority of species. This alignment highlights the highly conserved regions surrounding the conserved cysteine residues (*). Species abbreviations used are as follows: App, *A. pleuropneumoniae*; Pa, *P. aeruginosa*; Vc, *Vibrio cholerae*; Sp, *Shewanella putrefaciens*; Lp, *Legionella pneumophila*; Xc, *X. campestris*; Cc, *Caulobacter crescentus*; Bs1, *B. subtilis* YklA (OhrA); Ba, *Bacillus anthracis*; Bs2, *B. subtilis* YkzA (OhrB); Dr, *Deinococcus radiodurans*; Sa, *Staphylococcus aureus*; Ef, *E. faecalis*; Mp, *Mycoplasma pneumoniae*; Mg, *Mycoplasma genitalium*.

5×10^9 CFU of a clone containing the pTF86 vector only does not result in disease symptoms in the pig (13). When lung tissue isolated from a pig infected with APP233/pTF86 was examined, there were no regions of necrosis or of *lux* expression (Fig. 1).

Cloning and characterization of full-length *ohr*. The nucleotide sequence of *iviK* was used to design inverse PCR primers to clone the full-length *ohr* gene. A 2.5-kb PCR product was obtained and cloned into pGEM-T to form pinKE. This insert was sequenced, and alignment of this sequence with that of *iviK* demonstrated a contiguous ORF of 432 bp encoding 143 amino acids. This ORF was identified based on sequence homology as *ohr* (Fig. 2). Primers were designed to amplify the entire *ohr* gene plus 360 bp of upstream sequence. This PCR product was cloned into the pGZRS18 vector to form pGeohr.

A potential ribosome binding site (AAGGA) at 12 bp upstream of the start codon was identified. Potential transcriptional terminators flanking the ORF were identified using the GCG Stemloop program (Genetics Computer Group [Madison, Wis.] program manual for the Wisconsin package, 8th ed.). The predicted protein sequence was compared to finished and partially finished bacterial genomes deposited in the Microbial Genomes BLAST databases using the BLAST programs (National Center for Biotechnology Information). The ORF had highest homology to Ohr from *P. aeruginosa* with 62% similarity and 48% identity when examined using BLAST pairwise homology (29, 40). Proteins with strong homology to Ohr were identified from 14 species of bacteria and were aligned to identify regions of identity and similarity (Fig. 2). This alignment shows two regions of high conservation that center around conserved cysteines, with the sequence outside of these regions lacking extensive similarity. Analysis of the protein using PSORT to predict cellular localization suggested that Ohr was a cytoplasmic protein (26).

The region upstream of Ohr was also used to search databases at both the nucleotide and protein levels using the BLAST programs (National Center for Biotechnology Information), and no matches of significant homology were detected. The upstream region contains several small ORFs, all of ≤50 amino acids, which lack significant homology to known proteins.

Distribution of *ohr* among serotypes correlates with organic peroxide resistance. To determine if *ohr* was present in all 12 serotypes of *A. pleuropneumoniae*, Southern blotting was per-

formed with *Eco*RI-digested genomic DNA from all 12 serotypes as the target and with the use of the full-length *ohr* gene from *A. pleuropneumoniae* serotype 1 to construct the probe. Hybridizing bands were seen with genomic DNA from *A. pleuropneumoniae* serotypes 1, 9, and 11 (Fig. 3A) under stringent conditions, whereas no hybridization was seen with genomic DNA from *A. pleuropneumoniae* serotypes 2 to 8, 10, or 12 even under relaxed conditions.

Sensitivity to oxidative stress reagents for each of the 12 *A. pleuropneumoniae* serotypes was examined using a disk inhibition assay. Overnight cultures were added to top agar and overlaid with disks saturated with hydrogen peroxide, CHP, or the superoxide generator paraquat. After 22 h, the zones of growth inhibition were recorded as a measure of the sensitivity of each serotype to the oxidative stress imposed. Of the 12 serotypes, 11 showed equivalent sensitivities to hydrogen peroxide and paraquat (Fig. 3C and D). The exception was serotype 6, which showed a significantly larger zone diameter ($P < 0.01$) with all three forms of oxidative stress, suggesting a decreased resistance to oxidative stress reagents in general for this serotype. Serotypes 2 to 8, 10, and 12 showed sensitivity to CHP similar to that seen with hydrogen peroxide and paraquat (Fig. 3B to D). In contrast, serotypes 1, 9, and 11 showed a significantly reduced zone of growth inhibition upon incubation with CHP, reflecting an increased resistance to oxidative stress caused by organic peroxides (Fig. 3B). This increased resistance of serotypes 1, 9, and 11 to CHP was highly statistically significant ($P < 0.0001$), and no statistically significant difference was seen for these serotypes in response to incubation with hydrogen peroxide or paraquat. This increased resistance to CHP, but not to hydrogen peroxide or paraquat, correlated with the presence of the *ohr* gene as shown by Southern blotting (Fig. 3A).

Induction of *ohr* in response to oxidative stress. To characterize the expression of *ohr* in response to different oxidative stress reagents, induction studies were performed with wild-type *A. pleuropneumoniae* serotype 1. The analysis was performed with the wild-type strain in order to decrease the oxidative stress imposed on the cultures due to the presence of riboflavin in the medium that is necessary for growth of the APP233 strain. APP225/piviK, which contained the *ohr* promoter-*luxAB* fusion, was induced in microtiter plates with paraquat, CHP, or hydrogen peroxide. Addition of CHP, at 125, 300, or 1,000 μM, resulted in a rapid increase in *lux* expression in comparison to the noninduced control (Fig. 4). Neither paraquat nor hydrogen peroxide caused any induction, and the level of *lux* expression was equivalent to that seen in the absence of oxidative stress (Fig. 4). The concentrations of oxidative stress reagents were selected as the maximal sublethal dose (data not shown).

Comparison of induction of *ohr* in *A. pleuropneumoniae* serotype 1 and *A. pleuropneumoniae* serotype 5. We compared the expression of the *ohr*::*lux* fusion by using APP225/piviK (serotype 1) and APP227/piviK (serotype 5) during normal growth in culture and under induction by CHP. A growth curve was determined for each serotype to evaluate the expression level of *ohr* during growth in an aerated broth culture. The expression of *ohr* for both *A. pleuropneumoniae* serotype 1 and *A. pleuropneumoniae* serotype 5, as measured by luciferase activity, remained constant during normal growth, with the increase

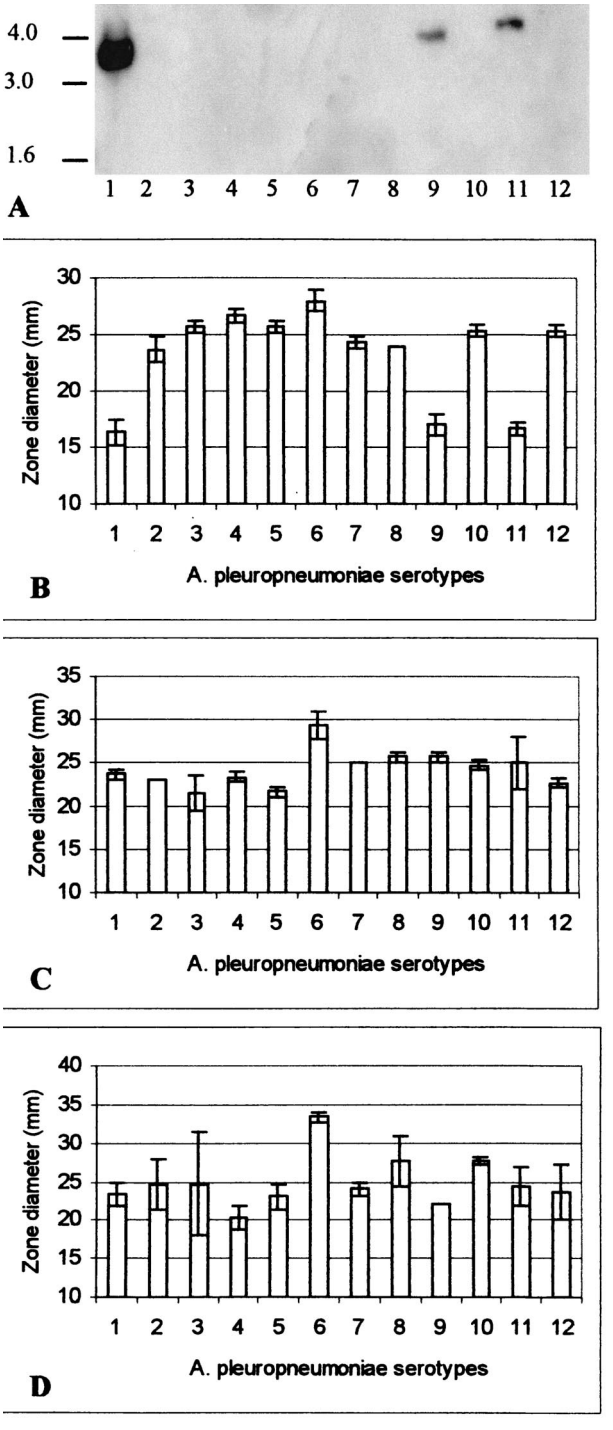

FIG. 3. Correlation of *ohr* presence with resistance to organic hydroperoxides. The presence of the gene was determined by Southern blotting, under high-stringency conditions, of genomic DNA with the intact *ohr* gene as a probe (A). Molecular size standards (in kilobases) are shown on the left. Each lane in panel A indicates a different serotype. The resistance of each of the 12 *A. pleuropneumoniae* serotypes to oxidative stress agents was determined by measuring the diameter of the zone of growth inhibition due to CHP (B), hydrogen peroxide (C), and paraquat (D). The data presented are the averages of three experiments, with the standard deviations shown by error bars. The differences in the zone diameters, after incubation with CHP, seen with serotypes 1, 9, and 11, in comparison to the remaining serotypes, are statistically significant ($P < 0.0001$).

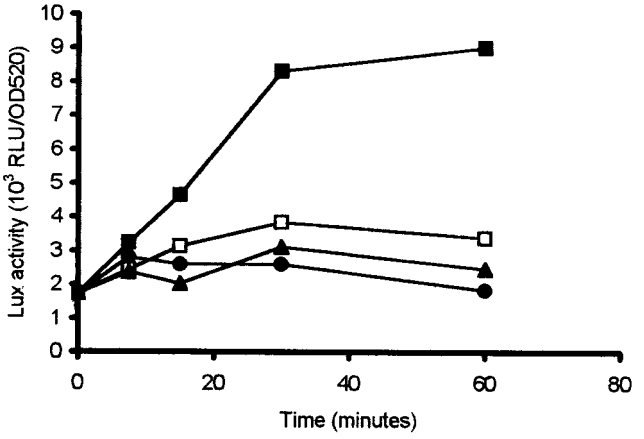

FIG. 4. Induction of the cloned *ohr* promoter in *A. pleuropneumoniae* serotype 1 (APP225/piviK) by various oxidative stress reagents. CHP (1 mM; ■), paraquat (50 μM; ●), and hydrogen peroxide (56 μM; ▲) were added as oxidative stress reagents, and Lux activity, expressed as RLU per OD_{520} unit, was measured. Lux activity was also measured in control cultures to which no inducing agent was added (□). Data presented are from a representative experiment. Trends were identical in all experiments.

over time directly correlated with the increase in total cell number (data not shown). The expression level was independent of serotype, with both serotypes showing low but equal expression levels during normal growth in the absence of inducers. This expression level of luciferase in the wild-type strains of *A. pleuropneumoniae* serotype 1 and *A. pleuropneumoniae* serotype 5, which averaged 1,000 RLU/OD unit, is greater than that seen for the *A. pleuropneumoniae* serotype 1 riboflavin mutant (APP233), which was never higher than 200 RLU/OD unit. Expression of luciferase from the pTF86 vector alone, with no insert, showed minimal expression of <50 RLU/OD unit in both serotypes (data not shown).

Expression of *ohr* under inducing conditions was examined for both *A. pleuropneumoniae* serotype 1 and *A. pleuropneumoniae* serotype 5. Induction of *ohr* in response to incubation with CHP was seen only in *A. pleuropneumoniae* serotype 1, not in *A. pleuropneumoniae* serotype 5 (Fig. 5). APP225/piviK showed rapid induction of expression, as measured by Lux assay with a twofold increase within 10 min post-exposure to CHP. Lux activity increased over time, with maximal levels detected between 30 and 60 min after induction. In contrast, APP227/piviK showed no increase in *lux* expression in response to CHP and maintained a level of expression slightly greater than that of the vector-only control. These data suggest that incubation with CHP does not cause induction of *ohr* in *A. pleuropneumoniae* serotype 5.

These induction data were confirmed through a functional assay of Ohr enzymatic activity. A xylenol orange colorimetric assay was used to determine the concentration of CHP after incubation of CHP with bacteria. Each serotype was grown in broth and then diluted into fresh media containing 125 or 300 μM CHP followed by incubation for 30 min to allow for induction. After this induction period, 125, 300, or 600 μM CHP was added and the rate of CHP degradation was measured. APP225, *A. pleuropneumoniae* serotype 1, showed significant Ohr activity, as measured by the rate of CHP degradation, in the absence of induction. Induction of *A. pleuropneumoniae* serotype 1 for 30 min with either 125 or 300 μM CHP resulted in an approximately twofold increase in enzymatic activity, which correlated well with the increase in *ohr* expression as measured by Lux activity (Fig. 5 and 6).

In contrast, neither APP227 (*A. pleuropneumoniae* serotype 5), APP227/pGZRS18, nor APP227/pGeohr showed significant Ohr activity in the absence of induction, and no increase in activity was evident under inducing conditions. These results correlate with and confirm the lack of expression of *ohr* in APP227 as measured by Lux activity (Fig. 5 and 6). The assays performed with APP227 showed that CHP concentrations of ≥300 μM CHP were lethal to the cells, and thus, induction and assays were performed at 125 μM CHP.

Evaluation of the mRNA start site in serotype 1 under CHP induction. Primer extension was performed using mRNA isolated from APP225/piviK that had been induced with CHP. The major transcriptional start site was located 31 bp upstream of the *ohr* start codon (Fig. 7). A −10 region (TAAAAT) was identified 6 bp upstream of the transcription start site. However, no −35 region similar to that found in *A. pleuropneumoniae* housekeeping genes (TTRAA, where R is A or G) could be identified (8). In the region in which a −35 site would be expected to exist, a SoxS binding motif (ACCGCAT) was found (34). This proposed SoxS binding motif is an exact match for the previously published *E. coli* SoxS binding motif (AnnGCAY) (34). Primer extension under noninducing conditions was also performed using sixfold more RNA. A primer extension product could not be detected under these noninducing conditions (data not shown).

DISCUSSION

Using the IVET system that we developed to facilitate identification of *A. pleuropneumoniae* genes involved in the pathogenesis of disease, we identified 42 unique promoter clones that were specifically induced during infection of the natural swine host. In this study, we report the identification and characterization of one of these in vivo-induced clones, *ohr*, which encodes an organic

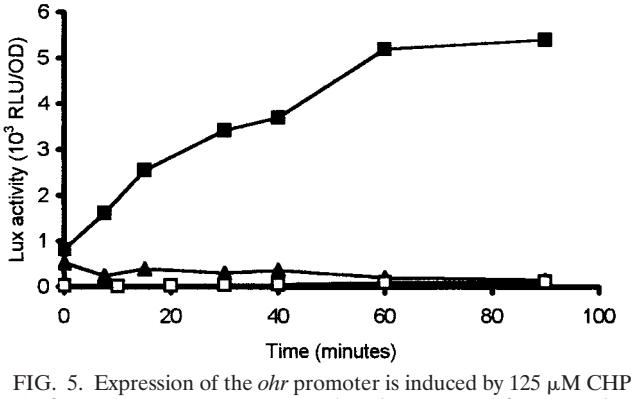

FIG. 5. Expression of the *ohr* promoter is induced by 125 μM CHP in *A. pleuropneumoniae* serotype 1 and not in serotype 5. *lux* expression driven by the *ohr* promoter was measured over time in *A. pleuropneumoniae* serotype 1 (APP225/piviK) (■) and in *A. pleuropneumoniae* serotype 5 (APP227/piviK) (▲) in comparison to a vector-only control, APP225/pTF86 (□). Data presented are from a representative experiment. Trends were identical in all experiments.

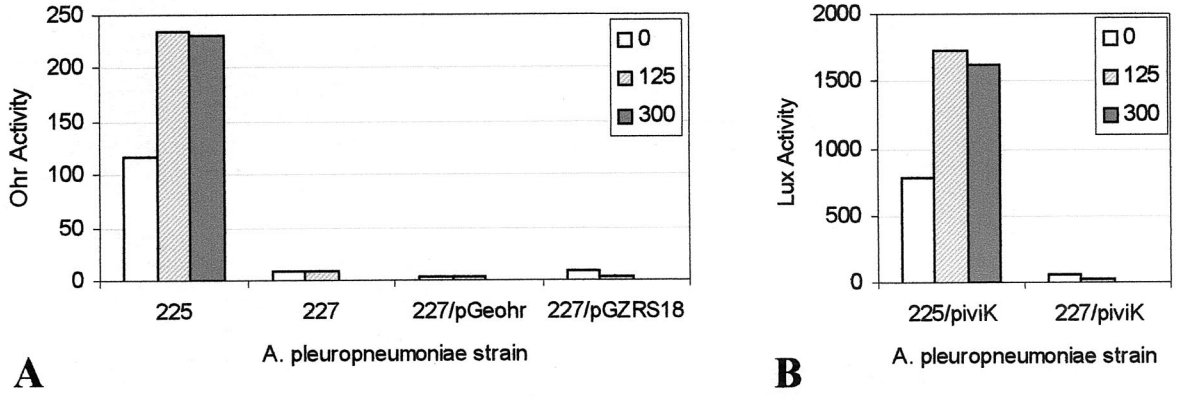

FIG. 6. (A) Ohr activity, expressed as micromoles of CHP degraded per minute, is induced by CHP in *A. pleuropneumoniae* serotype 1 (APP225) but not in serotype 5 containing the intact *ohr* gene plus promoter region (APP227/pGeohr). Controls include APP227 with no plasmid and APP227 containing the pGZRS18 shuttle vector only. For induction, CHP was added at final concentrations of 0, 125, or 300 μM to 1-ml broth cultures, which were held for 30 min at 35°C. Ohr activity was measured as degradation of CHP. (B) Lux activity, measured as RLU per OD unit, is similarly induced in APP225/piviK but not in APP227/piviK. Induction conditions for these assays were identical to those described for panel A. Data presented are from a representative experiment. Trends were identical in all experiments.

hydroperoxide reductase that we hypothesize is involved in protection from oxidative stress encountered during infection.

Enzymes responsible for conferring enhanced resistance to oxidative stress encountered during infection of the respiratory tract are potentially important virulence factors for organisms that cause pneumonia, such as *A. pleuropneumoniae* (6, 46). The mechanisms by which *A. pleuropneumoniae* causes disease lead to an environment filled with oxygen radicals (21, 27). Upon infection of the porcine lung by *A. pleuropneumoniae*, the host immune response to bacterial cell components, such as lipopolysaccharide, triggers an influx of inflammatory cells, particularly neutrophils, which are intended to limit the bacterial infection. Within this environment, *A. pleuropneumoniae* produces hemolysins and cytotoxins, in the form of RTX (repeats in toxin) toxins. These pore-forming RTX toxins secreted by *A. pleuropneumoniae* insert themselves into eukaryotic cell membranes and cause lysis and cell death of neutrophils and macrophages, which in turn release phagocyte contents such as oxygen radicals in the form of peroxides and superoxides (7, 11, 34). To survive in this environment, *A. pleuropneumoniae* likely requires enzymes that allow the bacteria to evade or detoxify these oxygen radicals. *A. pleuropneumoniae* has previously been shown to produce several enzymes involved in response to oxidative stress, including catalase and two separate types of superoxide dismutase (19, 21). This is the first report that this pulmonary pathogen produces an additional oxidative stress protectant, an organic hydroperoxide reductase, and the first demonstration that this type of enzyme can be specifically induced in vivo during the course of infection.

Ohr enzymes have been recently described for *X. campestris*, *P. aeruginosa*, *E. faecalis*, and *B. subtilis* and have been shown to be important in the survival of these bacteria when exposed to oxidative stress in vitro, although Ohr has not been previously implicated in virulence (12, 24, 29, 33). The *ohr* gene from these organisms exhibits a pattern of expression similar to that of *A. pleuropneumoniae ohr*. In each of these organisms, *ohr* is induced specifically in response to organic hydroperoxides, with little or no induction in response to hydrogen peroxide and superoxide (12, 24, 29, 33). This pattern of induction is distinct from that seen with *ahpC*, which encodes Ahp (alkyl hydroperoxide reductase), a second class of organic hydroperoxide reductases found in many bacterial species, including *E. coli*, *Salmonella enterica* serovar Typhimurium, *B. subtilis*, and *P. aeruginosa* (1, 4, 30, 37). Ahp enzymes are induced by hydrogen peroxide and organic peroxides but not superoxides (30, 34). We identified putative Ohr sequences, based on homology to these five identified Ohr proteins, from nine additional species (Fig. 2), although not from any members of the family *Enterobacteriaceae*. The predicted Ohr proteins from these 14 species share two regions of strong homology that flank conserved cysteines, which may be responsible for the reduction of peroxides to the corresponding alcohol (10). Similar conserved cysteines are seen in Ahp enzymes, which are functionally similar to Ohr enzymes but not closely related at the DNA level or protein level.

When we examined the type strains of the 12 known serotypes of *A. pleuropneumoniae* for the presence of *ohr* by Southern blotting, we were able to detect an *ohr* homologue only in *A. pleuropneumoniae* serotypes 1, 9, and 11 and not in serotypes 2 to 8, 10, and 12. The intensity of the band in *A. pleuropneumoniae* serotype 1 was greater than that of either serotype 9 or serotype 11, suggesting that the gene in serotypes 9 and 11 was not completely homologous to that of serotype 1, from which the probe was prepared. This distribution correlates with what is known about the relatedness of *A. pleuropneumoniae* serotypes. Serotypes 1, 9, and 11 are closely related to one another, having essentially the same lipopolysaccharide O-antigen chain, the same complement of RTX toxins produced, and an identical genotype for one of these toxins, *apxIA* (17, 31). *A. pleuropneumoniae* serotypes 1 and 9 have also been shown previously to be closely related to one another and distinct from serotypes 2 to 8 by multilocus enzyme electrophoresis (25). The differential distribution of *ohr* among the serotypes may reflect an evolutionary relatedness of these serotypes.

The presence of *ohr* among the 12 serotypes of *A. pleuropneumoniae* correlates with resistance to oxidative stress reagents. Serotypes 2 to 8, 10, and 12, which do not contain an *ohr* gene, were equally sensitive to all types of oxidative stress

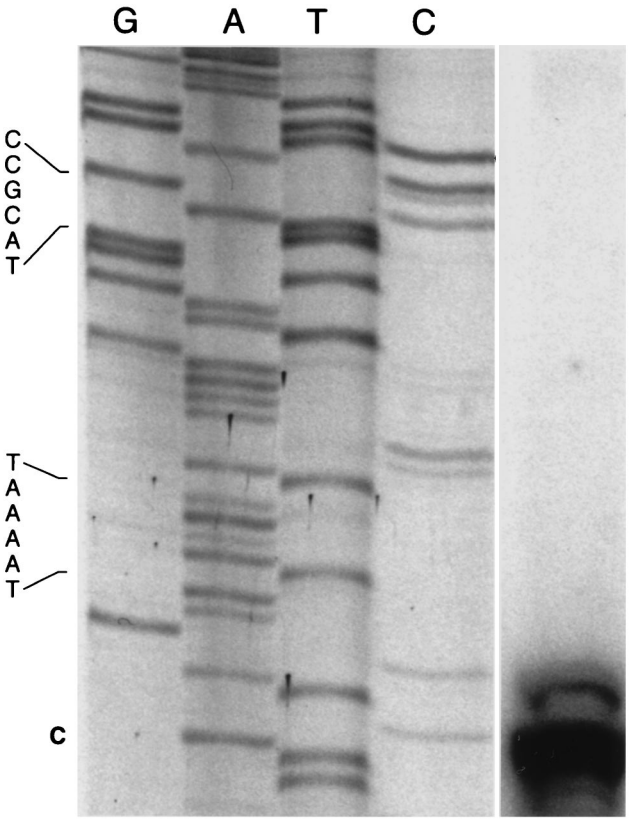

FIG. 7. Primer extension analysis of APP225/piviK induced with 1 mM CHP. The sequencing reactions and the primer extension reaction were performed with an identical primer. The lane on the right contains the primer extension product. The transcriptional start site is labeled in bold ("C" at bottom). The −10 region (TAAAAT) and the potential SoxS binding motif (CCGCAT) are indicated on the left. An intervening region between the sequencing ladder and the primer extension product was removed, without alteration of alignment.

agents tested, as judged by the zone of growth inhibition upon exposure to CHP, paraquat, and hydrogen peroxide (Fig. 3). In contrast, serotypes 1, 9, and 11 were significantly less sensitive to growth inhibition by CHP than were the other serotypes but were similar to the other serotypes in sensitivity to hydrogen peroxide and paraquat. *A. pleuropneumoniae* serotypes 1, 9, and 11 showed an increased resistance to CHP, but not to hydrogen peroxide or superoxide, that correlates with the presence of the *ohr* gene (Fig. 3).

The increased resistance to organic peroxides but not to other forms of oxidative stress seen for *A. pleuropneumoniae* serotypes 1, 9, and 11 correlates well with data on the induction of the *ohr* promoter by various stress reagents in *A. pleuropneumoniae* serotype 1. Induction of *ohr* was measured by luciferase assays using the *ohr* promoter fused to *luxAB* reporter genes and by assay of Ohr enzymatic activity via colorimetric detection of CHP degradation. With both of these methods, *ohr* expression in *A. pleuropneumoniae* serotype 1 was induced by CHP but not by either hydrogen peroxide or paraquat (Fig. 4 and 6).

We cloned both the intact serotype 1 *ohr* gene plus promoter region and an *ohr*::*luxAB* gene fusion into *A. pleuropneumoniae* serotype 5, which lacks *ohr*. During growth in broth under noninducing conditions, serotype 1 and serotype 5 showed low but equivalent expression as assayed by *lux* expression. However, while *A. pleuropneumoniae* serotype 1 is rapidly induced upon exposure to CHP, this induction is not seen with *A. pleuropneumoniae* serotype 5, either as increased expression of luciferase or as increased Ohr enzymatic activity (Fig. 5 and 6). We conclude that *A. pleuropneumoniae* serotype 5 not only does not contain a wild-type *ohr* gene but also is unable to respond to exposure to CHP by induction of the cloned serotype 1 *ohr* gene. This suggests that *A. pleuropneumoniae* serotype 5 may lack not only the *ohr* gene itself but also an additional gene(s) necessary to increase the expression of *ohr* in *A. pleuropneumoniae* serotype 1.

Multiple regulators that respond to oxidative stress have been identified for other prokaryotes, but none have as yet been identified for *A. pleuropneumoniae*. Three of the most well-studied regulators of oxidative stress responses in bacteria are OxyR, PerR, and SoxR (2, 34). OxyR, which has been identified for many gram-negative bacteria, is activated by exposure to peroxide, induces expression of *ahpC* (alkyl hydroperoxide reductase) and catalase, and also represses its own expression (30, 42). In both *X. campestris* and *P. aeruginosa*, *ohr* expression was not altered by lack of OxyR (24, 29). In many gram-positive organisms, *ahpC* and catalase are regulated by PerR, a homologue of the ferric uptake regulator Fur, which is functionally analogous to OxyR (2, 5, 34). Both of these regulators have known binding motifs that were not found in the *A. pleuropneumoniae ohr* promoter region (5, 42). SoxR, a transcription factor that is activated by superoxide, induces expression of a second transcription factor, SoxS, which in turn induces expression of superoxide-regulated genes such as *sodA*. SoxRS has also been shown elsewhere to regulate *ahpC* in some organisms but has not been demonstrated to be activated by peroxides (22, 32, 34). In *P. aeruginosa*, *ohr* induction was not affected by mutations in SoxR (29).

Recently, a novel transcriptional regulator, *ohrR*, has been described for *B. subtilis* and *X. campestris* (12, 39). In both organisms, *ohrR* is located immediately upstream of the *ohr* gene and encodes a 17-kDa peptide that is a member of the MarR family of transcriptional repressors. Expression of *B. subtilis ohrA* and *X. campestris ohr* is induced by organic peroxides and repressed by OhrR (12, 39). In *B. subtilis*, a 15-bp inverted repeat sequence overlapping the −10 promoter element is required for OhrR-mediated repression of the *ohrA* gene (39). No such repeat was found in the *X. campestris ohrR-ohr* intragenic region.

Analysis of 507 bp upstream of *A. pleuropneumoniae ohr* showed no evidence for an *ohrR* homologue. When we examined the promoter region of *A. pleuropneumoniae ohr* for potential regulatory sequences, we identified a potential SoxS box but no PerR or OxyR binding sequences or potential inverted repeats, such as that seen with the *B. subtilis* OhrR binding motif. SoxS in other organisms does not respond to organic peroxides, and the *A. pleuropneumoniae ohr* gene was not induced by superoxide generators. Our results suggest that a novel regulator or regulatory sequence is responsible for induction of *ohr* in *A. pleuropneumoniae* and that this novel regulator exists in *A. pleuropneumoniae* serotype 1 and not in serotype 5. Further studies are in progress to identify this regulatory molecule and to evaluate the role of Ohr in pulmonary infection caused by *A. pleuropneumoniae*.

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