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Genetic and Biochemical Analyses of *Actinobacillus pleuropneumoniae* Urease

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The urease gene cluster from the virulent *Actinobacillus pleuropneumoniae* serotype 1 strain CM5 was cloned and sequenced. The urease activity was associated with a 6.3-kbp region which contains eight long open reading frames (ORFs). The structural genes, *ureABC*, are separated from the accessory genes, *ureEFGD*, by a 615-bp ORF of unknown function, *ureX*. Homologies were found with the structural and accessory urease gene products of *Haemophilus influenzae* and, to a lesser extent, with those of other organisms. The urease enzyme subunits had predicted molecular masses of 61.0, 11.3, and 11.0 kDa, and the size of the holoenzyme was estimated to be 337 ± 13 kDa by gel filtration chromatography. Urease activity was maximal but unstable at 65°C. In cell lysates, the *A. pleuropneumoniae* urease was stable over a broad pH range (5.0 to 10.6) and the optimal pH for activity was 7.7. The K_m was 1.5 ± 0.1 mM urea when it was assayed at pH 7.7. The low K_m suggests that this enzyme would be active in the respiratory tract environment, where urea levels should be similar to those normally found in pig serum (2 to 7 mM).

Actinobacillus pleuropneumoniae is the causative agent of fibrinohemorrhagic pneumonia and pleuritis in pigs of all ages (34, 42). The clinical signs associated with acute disease include high fever, coughing, dyspnea, anorexia, ataxia, and severe respiratory distress with cyanosis. The pathogenesis of the disease is multifactorial, with RTX toxins, lipopolysaccharide, capsule, and certain outer membrane proteins thought to contribute to virulence (10, 15). To date, vaccines based on these virulence factors have not been very effective, however, suggesting that additional components are important in the disease process.

Virtually all *A. pleuropneumoniae* field isolates are strongly urease positive (26). This enzyme catalyzes the hydrolysis of urea to produce ammonia and carbon dioxide and is known to be a virulence factor in certain gastroduodenal and urinary tract pathogens (4, 29). Ammonia released during urea hydrolysis is responsible for many of the effects of urease activity. Ammonia is cytotoxic for gastric epithelial cells and enhances neutrophil-dependent cell damage (27, 45, 47). Ammonia has also previously been implicated in the inactivation of the fourth component of the complement cascade (2). In addition, the urease enzyme is chemotactic and can activate polymorphonuclear leukocytes and monocytes (24, 25).

Bacterial ureases are typically comprised of three structural subunits (α, β, and γ) that are encoded by three genes, *ureABC* (29). Although the details of organization differ, the genes for accessory proteins UreE, UreF, UreG, and UreD (UreH in *Helicobacter pylori* and *Haemophilus influenzae* [9, 19]) are linked to *ureABC* (7, 18, 19, 21, 23, 29, 31). In *Helicobacter pylori* and *Bacillus* sp. strain TB-90, an additional gene (*ureI* and *ureH*, respectively) is present in the urease gene cluster, but their roles are not certain (19, 23). In some organisms, such as *Morganella morganii* (43), urease expression is constitutive. In others, it is regulated by low levels of nitrogen (e.g., *Klebsiella aerogenes* [33]), by the presence of urea (e.g., *Proteus* and *Providencia* spp. [39]), or by pH (*Streptococcus salivarius* [44]).

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Quite a few respiratory tract pathogens produce urease (13); however, there have been few studies of the role of this enzyme in the disease process (30, 37). To investigate the role of urease in the pathogenesis of *A. pleuropneumoniae*, we cloned and sequenced the genes responsible for urease production and characterized urease activity in *A. pleuropneumoniae* cell lysates.

(A preliminary report of these findings was presented at the 77th Conference of Research Workers in Animal Diseases, Chicago, Ill., 1996.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. A spontaneously nalidixic acid-resistant derivative of the virulent *A. pleuropneumoniae* serotype 1 strain CM5 (38) was selected and designated CM5 Nal^r. Chromosomal DNA from CM5 Nal^r was used for cloning as well as for isolation of the urease holoenzyme. The organism was routinely grown on CAV-Nal agar (consisting of Trypticase soy agar [Difco Laboratories, Detroit, Mich.] supplemented with 5% heated sheep blood [80°C for 10 min], 0.01% NAD [wt/vol], and 20 μg of nalidixic acid per ml) or in BHIV-Nal (brain heart infusion broth [Difco] supplemented with 0.01% NAD and 20 μg of nalidixic acid per ml). *A. pleuropneumoniae* CM5 Nal^r was checked for urease activity by heavily inoculating urea agar (Difco) or by adding 1/10 volume of 10× urea base medium (Difco) to broth cultures. *Escherichia coli* HB101 and DH5-α, the recipients of recombinant plasmids, were grown on Luria-Bertani (LB) medium supplemented with the appropriate antibiotic (tetracycline, 15 μg/ml; ampicillin, 100 μg/ml). Recombinants were screened for urease activity on urea agar. All broth cultures were grown at 37°C with shaking at 200 rpm. Plate cultures of *A. pleuropneumoniae* were grown in the presence of 5% CO_2 at 37°C.

Urease preparation. CM5 Nal^r was grown overnight in 500 ml of BHIV-Nal. Cells were harvested by centrifugation at 8,000 × *g* for 10 min at 4°C and washed twice with 20 mM sodium phosphate buffer (SPB; pH 6.8). The pellet (4.5 g [wet weight]) was suspended in 20 ml of cold SPB, and cells were disrupted by three passes through a French pressure cell at 20,000 lb/in^2. The lysate was clarified by centrifugation at 10,000 × *g* for 10 min at 4°C, and the supernatant was then subjected to ultracentrifugation at 100,000 × *g* for 16 h at 4°C. After ultracentrifugation, the protein concentration of the supernatant was determined by the method of Bradford with a kit from Bio-Rad Laboratories (Richmond, Calif.).

Urease assay. Urease activity was measured with a urea nitrogen assay kit (catalog no. 640-A; Sigma Chemical Co., St. Louis, Mo.) which permits the detection of NH_3 by the Berthelot reaction (49). Briefly, 25 μl of sample was added to 1.225 ml of assay buffer containing 50 mM HEPES, 1 mM EDTA, and urea (100 mM for all assays, except for the determination of enzyme kinetics, where urea concentrations ranged from 0.78 to 50 mM). Unless otherwise stated, assays were performed at 23°C. At timed intervals, 25-μl aliquots were removed to wells of a microtiter plate containing 50 μl of phenol nitroprusside, and then 50 μl of alkaline hypochlorite was added to stop the reaction. The optical density at 550 nm was read with a microplate autoreader (Bio-Tek Instruments, Wi-

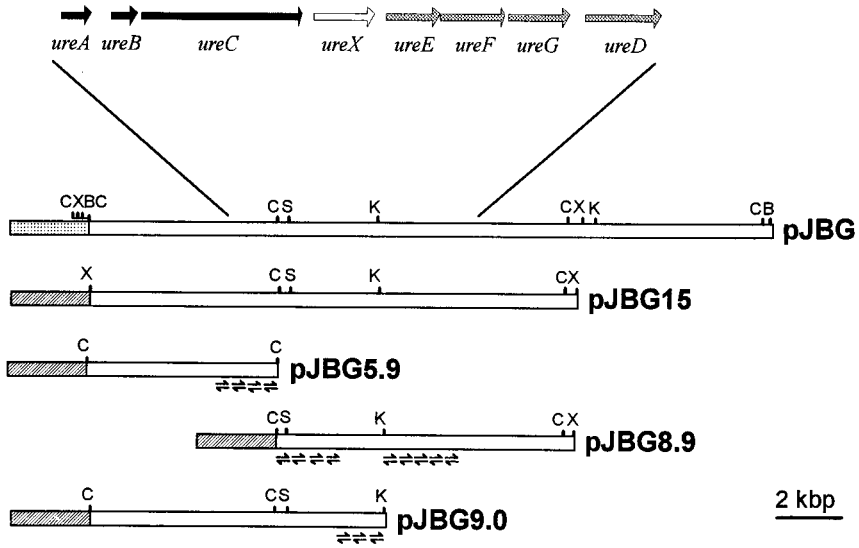


FIG. 1. Cloning and sequencing strategy for the *A. pleuropneumoniae* urease gene cluster. The pBluescript II KS+ (hatched) and pCP13 (stippled) vector sequences are not to scale. Forward and reverse sequencing reactions are indicated by arrows. C, *Cla*I; K, *Kpn*I; S, *Sph*I; X, *Xba*I.

nooski, Vt.) after incubation at room temperature for a minimum of 20 min. Urease activity was expressed in micromoles of NH_3 per minute per milligram (hereafter expressed in units per milligram). A standard curve of the ammonia concentration was prepared by using 1 to 10 mM ammonium chloride diluted in urease assay buffer. All samples and standards were analyzed in triplicate.

Biochemical analyses. The effects of temperature on the enzyme activities of cell lysates were determined by performing the urease assay at 23, 30, 37, 45, 56, and 65°C. The stabilities at different pHs were determined by incubating the enzyme in buffers ranging from pH 3.6 to 10.6 for 30 min and then assaying for urease activity in urease buffer (pH 7.5) at 23°C (48). The buffers used for pH stability experiments were 20 mM acetate (pH 3.6 to 5.6), 20 mM phosphate (pH 6 to 8), and 20 mM glycine-NaOH (pH 8.6 to 10.6). Kinetic assays were done with cell lysates in urease assay buffer with urea concentrations ranging from 0.78 to 50 mM. In order to determine the optimal pH for catalytic activity (48), kinetic assays were repeated with urease assay buffer (50 mM HEPES, 1 mM EDTA) with pHs ranging from 6.8 to 8.5.

Column chromatography. The size of the native urease was determined by gel filtration chromatography with a Superose 6 column (Pharmacia Biotech Inc., Baie d'Urfé, Quebec, Canada). The urease preparation was adjusted to 10 mg of total protein per ml in SPB with 100 mM KCl (to prevent aggregate formation). Fractions were eluted at a flow rate of 0.3 ml/min in SPB containing 100 mM KCl. Fractions (300 μl) were collected and assayed qualitatively for urease activity by incubating 25 μl of eluate in 250 μl of 1× concentration of urea base medium at 37°C for 1 to 2 h. The elution volume for urease was determined with three separate samples. The column was calibrated with blue dextran (2,000 kDa) for void volume and with the following protein molecular size standards: catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa).

Construction of genomic library. A gene library of CM5 Nal^r DNA was constructed in cosmid vector pCP13 by standard methods (40). Briefly, chromosomal DNA from CM5 Nal^r was partially digested with *Sau*3A. Fragments of ≥12 kbp, obtained by sucrose gradient fractionation, were ligated into the *Bam*HI site of pCP13 (6) and packaged in vitro (Packagene lambda DNA packaging system; Promega, Nepean, Ontario, Canada). Phage particles containing recombinant DNA were used to infect *E. coli* HB101, and transductants were selected by growth on LB agar supplemented with 15 μg of tetracycline per ml. One strongly urease-positive clone, pJBG91, was detected after subculture on urea agar.

Subcloning and nucleotide sequencing. The 21-kbp insert of pJBG91 was partially mapped by using single and double digests with *Cla*I, *Kpn*I, *Sph*I, and *Xba*I (Pharmacia Biotech Inc.). Selected fragments were subcloned into plasmid vector pBluescript II KS+ (Stratagene, Inc., La Jolla, Calif.) and electroporated into *E. coli* DH5-α. Transformants were selected by plating on LB agar supplemented with 100 μg of ampicillin per ml. Plasmid DNAs were isolated by using plasmid purification columns (QIAGEN Inc., Chatsworth, Calif.). Three subclones, pJBG5.9, pJBG8.9, and pJBG9.0, were used for sequencing the urease gene cluster (Fig. 1). Sequencing was carried out at GenAlyTiC (Dept. of Zoology, University of Guelph, Guelph, Ontario, Canada) with a DNA sequencer (model 377; Applied Biosystems) and a *Taq* dyedeoxy terminator cycle sequencing kit (Perkin-Elmer, Foster City, Calif.). Sequencing was initiated with universal primers to the M13 forward and reverse sequences within pBluescript II KS+, and oligonucleotide primers were synthesized (GIBCO BRL, Grand Island, N.Y.) to complete the sequencing of both strands.

The sequence data were compiled, edited, and analyzed by using the GeneRunner software package (version 3.04; Hastings Software Inc., Hastings on Hudson, N.Y.). Nucleotide and protein sequence homology searches were performed by using the BLAST program (1, 11) from the National Biomedical Research Foundation (National Institutes for Medical Research, Bethesda, Md.), and multiple-protein-sequence alignments were done with ALIGN (version 1.02; Scientific and Educational Software, State Line, Pa.). Enzyme kinetic data were calculated by using the MacCurveFit program (Kevin Raner Software, Mt. Waverley, Victoria, Australia).

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to GenBank and assigned accession no. U89957.

RESULTS

Urease activity. After ultracentrifugation, the cell lysate (in SPB) contained 11 mg of protein per ml and exhibited a urease activity on day 1 of 35 ± 1 U/mg when it was tested at 23°C in urease assay buffer (pH 7.5) with 100 mM urea. There was no loss of activity after 84 days when the lysate was stored undiluted at −90°C (109% of original activity). Urease activity declined by approximately half after storage at −20°C (68%) and 4°C (50%) for 33 days.

Enzyme kinetics. The rate of urea hydrolysis was quantitated at 23°C in urease assay buffer (pH 7.5) with urea concentrations ranging from 0.78 to 50 mM. Under the assay conditions used, urease activity in cell lysates exhibited first-order kinetics. The reciprocal plot of 1/S versus $1/V_o$ revealed a K_m of 1.7 ± 0.2 mM urea and an activity of 29 ± 1 U/mg. Urease activity was not inducible by growth in the presence of 0.1% (wt/vol) urea.

Effects of pH on activity. The urease activity of the cell lysate was stable over a broad range of pHs. Greater than 80% of maximum activity was retained for 30 min at 23°C when urease was exposed to buffers with pHs ranging from 5.0 to 10.6. Exposures to buffers with pH values of less than 5.0 led to a rapid loss of activity (62% of maximum at pH 4.6, 24% of maximum at pH 4.0, and 13% of maximum at pH 3.6). The stability of urease activity after exposure to a buffer with a pH value of greater than 10.6 was not investigated.

When kinetic parameters were measured in urease assay buffer at different pHs, the K_m declined slightly, from 2.5 ± 0.2

TABLE 1. Urease activities of *A. pleuropneumoniae* cell lysates at selected temperatures[a]

Time (min)	Urease activity (U/mg)					
	23°C	30°C	37°C	45°C	56°C	65°C
20	22 ± 3	27 ± 1	33 ± 1	43 ± 1	48 ± 1	69 ± 2
40	22 ± 1	28 ± 1	33 ± 1	44 ± 3	50 ± 1	50 ± 1
60	22 ± 1	28 ± 1	32 ± 1	42 ± 1	48 ± 1	45 ± 1

[a] Samples were assayed for urease activity every 10 min (up to 60 min).

mM urea at pH 6.8 to 1.3 ± 0.1 mM urea at pH 8.5. Enzyme activity did not vary greatly when it was measured at pHs ranging from 6.8 (29 ± 1 U/mg) and 8.5 (28 ± 1 U/mg). The highest catalytic activity (33 ± 1 U/mg) was found at pH 7.7, where the enzyme had a K_m for urea of 1.5 ± 0.1 mM.

Effects of temperature on activity. The rate of urea hydrolysis increased with increasing temperature up to 65°C. However, the enzyme was unstable at 65°C, as indicated by the decrease in activity at 60 min compared to that at 20 min of incubation (Table 1).

Native molecular weight. An estimation of the native molecular weight of the *A. pleuropneumoniae* urease was obtained with a calibrated Superose 6 gel filtration column. When cell lysate was applied to the column, a single peak of urease activity was seen; it corresponded to a molecular mass of 337 ± 13 kDa.

Cloning and sequencing of the urease gene cluster. The cosmid library of *A. pleuropneumoniae* CM5 Nal^r chromosomal DNA was screened on urea agar. Cosmid pJBG91 from a strongly urease-positive clone was purified, and a partial restriction map was generated with enzymes *Cla*I, *Kpn*I, *Sph*I, and *Xba*I (Fig. 1). A 14.8-kbp *Xba*I fragment was subcloned into pBluescript II KS+, resulting in the Ure$^+$ plasmid pJBG15. The urease activity of this subclone was similar to that of *A. pleuropneumoniae* CM5. Three further subclones, pJBG5.9, pJBG8.9, and pJBG9.0, were all Ure$^-$.

Eight long open reading frames (ORFs), all translated in the same direction, were contained within a 6.7-kbp region spanned by pJBG5.9, pJBG8.9, and pJBG9.0. Seven of the ORFs have significant homologies with other structural (*ureABC*) and accessory (*ureEFGD*) urease genes and were named accordingly (Table 2). The highest homologies were seen with the *H. influenzae* urease genes. The 615-bp ORF, *ureX*, between the structural and accessory genes does not have homology with any reported gene sequence.

The *A. pleuropneumoniae* urease genes encode structural subunits with predicted molecular weights of 11,000 (UreA), 11,300 (UreB), and 61,800 (UreC) and accessory gene products with predicted molecular weights of 21,700 (UreE), 25,400 (UreF), 23,100 (UreG), and 30,200 (UreD). The *ureX* gene could encode a polypeptide with a molecular weight of 23,500.

All eight ORFs have predicted ATG start codons and are preceded by sites similar to the *E. coli* ribosome-binding site consensus sequence (3). Putative σ-70 promoter elements (12) are present upstream of *ureA*, *ureX*, and *ureE* (Fig. 2). No long ORFs were found within ≥500 bp upstream of *ureA*, and no homology to the urease regulatory gene (*ureR*) was detected within this region. Several common histidine residues (corresponding to His-96 in UreA, His-39 and His-41 in UreB, and His-134, His-136, His-219, His-246, His-312, His-320, and His-321 in UreC of *K. aerogenes*) are present in the *A. pleuropneumoniae* urease. Key residues previously shown to be involved in nickel metallocenter binding (i.e., those corresponding to Asp-360, Lys-217, and His-134, -135, -246, and -272 in *K. aerogenes*), as well as His-219, a putative substrate binding residue, and His-320, which probably serves as the general base for catalysis, are conserved in the *A. pleuropneumoniae* UreC (Fig. 2) (29, 35). The *A. pleuropneumoniae* UreE has a histidine-rich carboxy terminus (29), and an ATP- and GTP-binding sequence is present in the amino-terminal end of UreG (41).

DISCUSSION

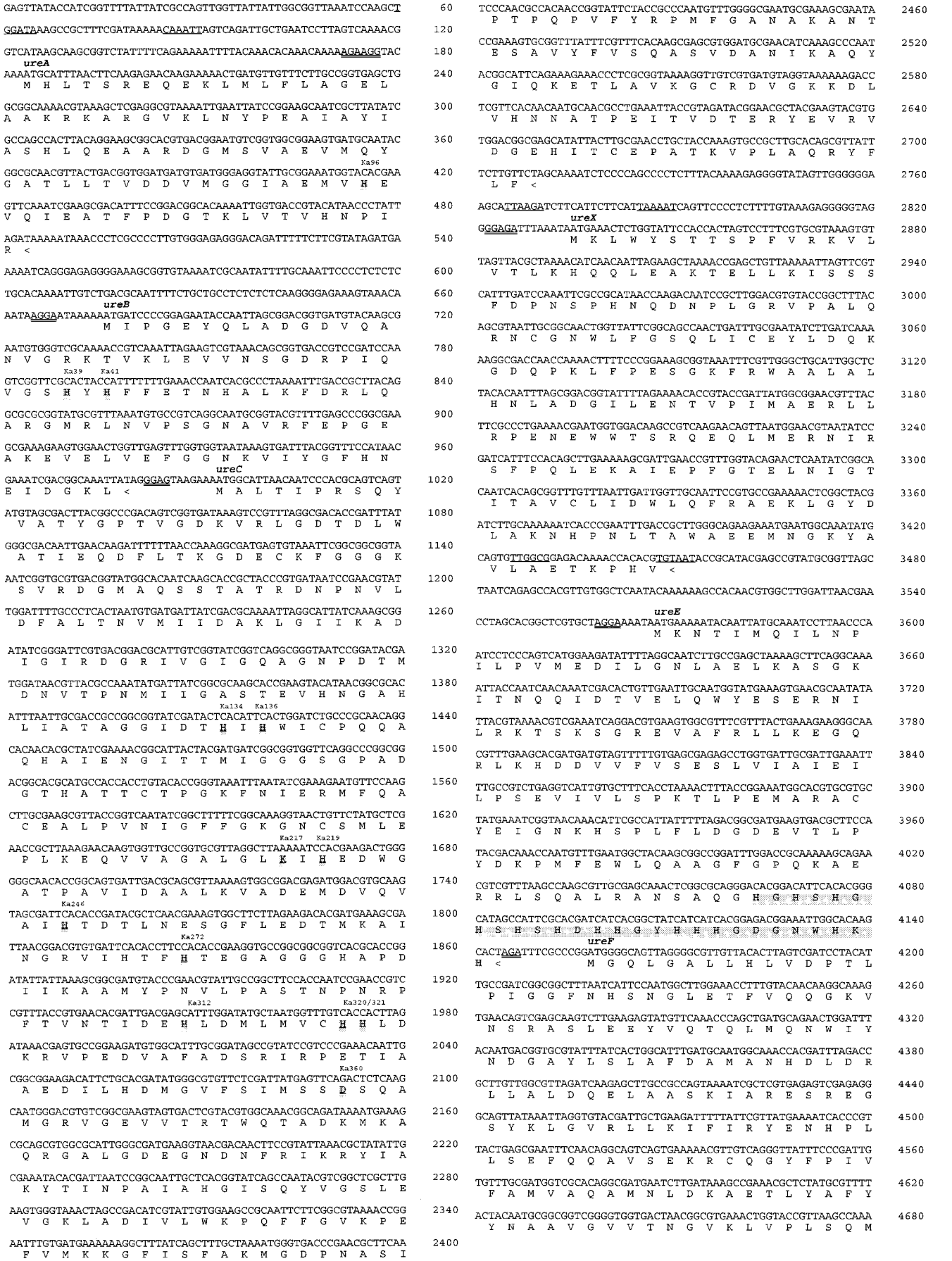
The *A. pleuropneumoniae* urease genes exhibit the general organization of other bacterial urease gene clusters (29). The first three ORFs encode the structural proteins (UreABC) from the smallest to the largest subunit, and the accessory proteins (UreEFGD) are encoded by four contiguous genes downstream. An additional ORF, *ureX*, lies between the structural and accessory genes. The *Helicobacter pylori* urease gene cluster also contains an additional ORF between the structural and accessory urease genes, *ureI* (5). A mutation of *ureI* leads to a urease-negative phenotype in *Helicobacter pylori* but not in *E. coli* containing the cloned *H. pylori* gene cluster (5, 8). Its precise role in urease expression is unclear, however.

The deduced amino acid sequences of the structural and accessory urease genes of *A. pleuropneumoniae* have their highest homologies with those of *H. influenzae*. The urease gene products of *A. pleuropneumoniae* and *H. influenzae* form a separate phylogenetic group which is quite divergent from other bacterial ureases (Table 2). The *A. pleuropneumoniae* urease structural subunits are predicted to contain all of the conserved amino acids which have previously been shown to be important in the expression of urease activity, including ligands to the nickel metallocenter, a possible substrate binding residue, and the likely general base required for catalysis (Fig. 2) (16, 29, 35, 46). As with other bacteria, the accessory proteins of the *A. pleuropneumoniae* urease, with the exception of UreG, are less well conserved than are the structural subunits (Table 2). The *A. pleuropneumoniae ureE* gene product has a

TABLE 2. Comparison of the deduced amino acid sequences of the *A. pleuropneumoniae* urease genes with those of other bacterial urease genes

Organism (GenBank accession no.)	% Identity						
	ureA	*ureB*	*ureC*	*ureE*	*ureF*	*ureG*	*ureD* (*ureH*)[a]
H. influenzae (U32736, L42023)	96	86	87	85	67	95	(70)
Bacillus sp. strain TB-90 (D14439)	67	55	62	21	34	60	24
Helicobacter pylori (X57132, M60398, M84338)	64	53	62	28	34	66	(29)
K. aerogenes (M55391, M36068)	57	60	66	25	27	60	21

[a] Parenthetical data are for *ureH*.



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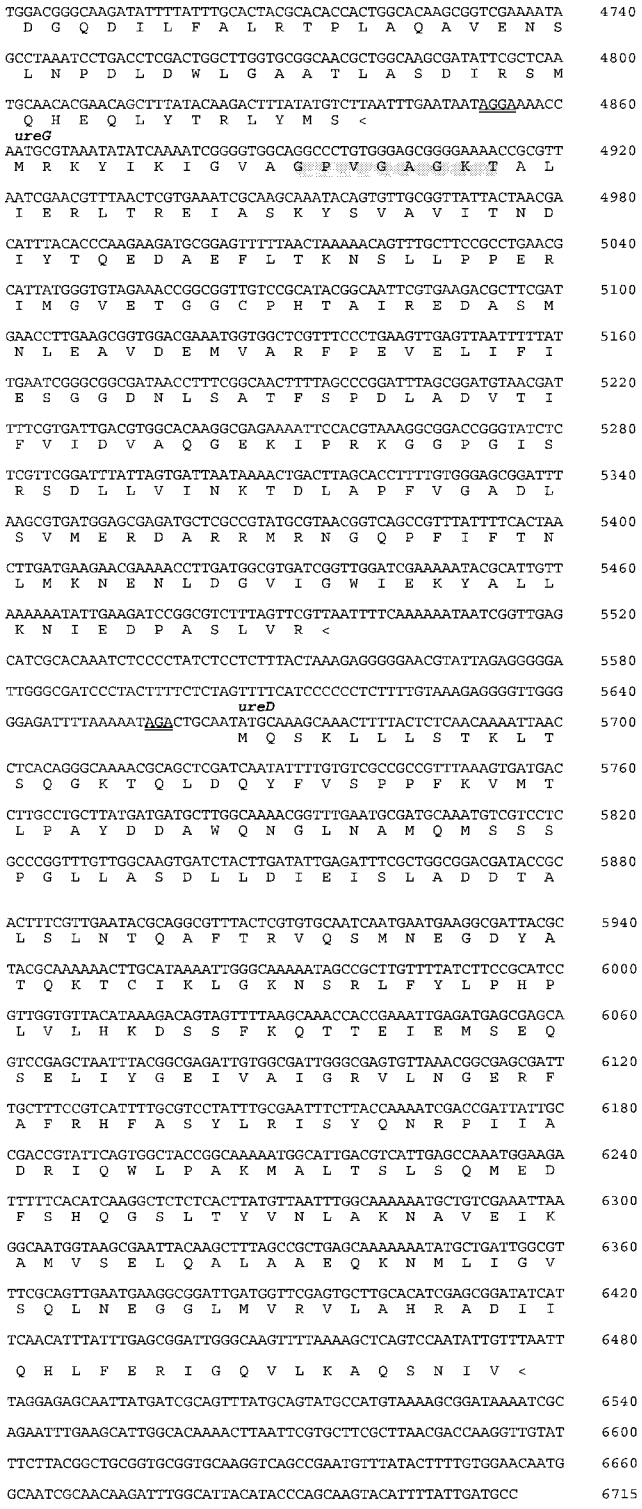

FIG. 2. Nucleotide and deduced protein sequences of the *A. pleuropneumoniae* urease gene cluster. Putative −35 and −10 promoter elements are underlined; Shine-Dalgarno-like sequences are indicated by double underlining; conserved amino acid residues, the histidine-rich tail in UreE, and the ATP- and GTP-binding motif in UreG are shaded. Ligands to the binickel center and other active-site residues are shaded and underlined. Stop codons are indicated by less-than signs (<).

histidine-rich carboxy terminus which is present in most ureases, and an ATP- and GTP-binding motif, found in all ureases studied to date, is present in UreG (20, 22, 29, 36, 41).

The native molecular mass of the *A. pleuropneumoniae* urease was estimated to be 337 ± 13 kDa, which is somewhat larger than the predicted size of an αβγ trimer. Mulrooney et al. (32) found that the native molecular weight of *Providencia stuartii* was similarly overestimated when cell extracts rather than purified enzyme were analyzed, presumably due to the association of additional proteins. In cell lysates, the *A. pleuropneumoniae* urease was quite stable and active. As with most other bacterial ureases, activity could be detected over a wide range of basic pHs (5.0 to 10.6) but was markedly reduced at pH 5 or less (29). In contrast to the *Helicobacter pylori* urease, the *A. pleuropneumoniae* urease appeared to be very stable (Table 1) (28). No activity was lost after storage at −90°C, and the enzyme was most active at temperatures of ≥65°C.

The urease activity in cell lysates of *A. pleuropneumoniae* (29 ± 1 U/mg at pH 6.8) was comparable to that found in cell lysates of *Helicobacter pylori* (36 ± 28 U/mg at pH 6.8), which is 2 times that of *Proteus mirabilis* and 10 times those of other urinary tract pathogens (28). The urease activity in *A. pleuropneumoniae* was not inducible by growth in the presence of 0.1% urea, and there was no evidence of a regulatory gene (*ureR*) upstream of *ureA*.

Like *Helicobacter pylori*, *A. pleuropneumoniae* is found in an environment where available urea is supplied from serum and where concentrations are low. It has previously been noted that the correlation between the K_m for a given urease and the environmental niche of the bacterium may be limited (28, 29). With the exception of *M. morganii*, the urease enzymes of urinary tract isolates belonging to the genera, *Proteus*, *Providencia*, and *Morganella*, that grow in urea-rich environments have K_ms ranging from 10 to 60 mM urea (17). In contrast, the *Helicobacter pylori* urease has a K_m for urea of 0.17 mM (14). The K_m of the *A. pleuropneumoniae* urease was 1.5 ± 0.1 mM urea, which allows this enzyme, like that of *Helicobacter pylori*, to operate under near saturating conditions despite low substrate concentrations.

The results of this study are consistent with our hypothesis that urease is important for the survival and/or pathogenesis of *A. pleuropneumoniae*. The expression of *A. pleuropneumoniae* urease requires the products of at least seven genes, spanning 6.3 kbp of DNA. Given its small (1.96-mbp) chromosome and the fact that urease-negative isolates are extremely rare, there appears to be strong selective pressure to maintain such a large amount of DNA. Furthermore, the fact that the enzyme has such a low K_m for urea suggests that it would be fully functional in the respiratory tract environment.

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